IMPORTANCIA FUNCIONAL DE LAS INTEGRINAS β1 COMO RECEPTORES DE ADHESION LINFOCITARIA EN LA ARTRITIS REUMATOIDE: IMPLICACIONES PATOGENICAS.

Memoria para optar al título de Doctor en Medicina presentada por:

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Reunido el Tribunal que suscribe en el día de la fecha, acordó calificar la presente Tesis Doctoral con la censura de APTO CUM LAUDE POR UNANIMIDAD

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A mis padres,

Angel y Silvina

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En primer lugar, quiero dar las gracias a todos mis compañeros de la Sección de Reumatología del Hospital de la Princesa, a los que me han acompañado durante todos estos años y a los que ya se han ido. Ellos me han enseñado, por encima de todo, lo que es ser un buen médico. Sin sus enseñanzas, apoyo y estímulo, hubiera sido imposible comenzar este trabajo y, mucho menos, acabarlo. Gracias especialmente a Armando Laffón, maestro y amigo, *inventor* incansable, por haberme inculcado, como a tantos otros, la necesidad de buscar más allá de lo ya escrito. Gracias al Dr. Sánchez-Madrid, que ha dirigido y estimulado constantemente mis modestos pasos en el campo de la investigación y la Inmunología. También quiero recordar, con especial cariño, a todos aquellos que han compartido conmigo el trabajo de tantas tardes, e incluso alguna madrugada, en el Laboratorio de Inmunología. Especialmente a Alicia, Federico, Isidoro y Antonio Postigo, gracias por hacer más cálido el ambiente de nuestro *chiringuito*.

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Y para terminar, GRACIAS a mi hermana Mertxe. Ella siempre está ahí.

Dª Rosario García de Vicuña ha desarrollado, bajo mi dirección, el trabajo titulado **"Importancia funcional de las integrinas β1 como receptores de adhesión linfocitaria en la Artritis Reumatoide: implicaciones patogénicas"**, realizado en los Servicios de Reumatología e Inmunología del Hospital de la Princesa,

El presente estudio describe en primer lugar, la distribución tisular amplia de varias integrinas β1 en la membrana sinovial reumatoide. Posteriormente, se demuestra la regulación *in vivo* de múltiples funciones adhesivas de linfocitos T, mediadas por integrinas β1, en eventos fundamentales en la patogenia de la enfermedad: adhesión a ligandos de células endoteliales, a la proteina de matriz extracelular fibronectina, y a sinoviocitos tipo fibroblasto de Artritis Reumatoide. Mediante ensayos de adhesión *in vitro*, se han investigado los diferentes ligandos que intervienen en estas interacciones así como posibles factores reguladores en cada proceso de adhesión. Finalmente, se describe por primera vez, la presencia de conformaciones activas de integrinas β1 en la Artritis Reumatoide y otras enfermedades inflamatorias crónicas, y la regulación de su expresión por mediadores fisiológicos como citoquinas y quimioquinas. El conocimiento de las bases moleculares de estas interacciones y su regulación abre la posibilidad para el desarrollo de nuevos enfoques terapéuticos dirigidos a detener el influjo de células inflamatorias a la sinovial. Así mismo, la actuación sobre algunas de las moléculas de adhesión estudiadas, podría interrumpir múltiples funciones efectoras de las células implicadas, que contribuyen decisivamente al daño tisular.

Por sus aportaciones e interés actual, este trabajo se estima merecedor de ser aceptado como TESIS DOCTORAL en la Facultad de Medicina (Departamento de Medicina) de la Universidad Autónoma de Madrid.

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Dr. Francisco Sánchez-Madrid
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CLAVE DE ABREVIATURAS

AcM	Anticuerpo monoclonal
AR	Artritis Reumatoide
CE	Célula endotelial
CD	Grupo de diferenciacion
ELAM	Molécula de adhesión endotelial para leucocitos
FN	Fibronectina
GM-CSF	Factor estimulador de colonias granulocito macrofágicas
HEV	Venulas de endotelio alto
ICAM	Molécula de adhesión intercelular
IFN	Interferon
Ig	Inmunoglobulina
IL	Interleuquina
LFA	Antígeno asociado a función de leucocitos
LS	Líquido sinovial
MA	Moléculas de adhesión
MEC	Matriz extracelular
MS	Membrana sinovial
RCT	Receptor de la célula T
SP	Sangre periférica
STF	Sinoviocito tipo fibroblasto
TNF	Factor de necrosis tumoral
VCAM	Molécula de adhesión vascular
VLA	Antígeno de activación tardía

INTRODUCCION

Múltiples fenómenos biológicos, normales y patológicos, son dependientes del contacto célula-célula o de la adherencia de células a componentes de la matriz extracelular . Los receptores celulares responsables de estos fenómenos complejos de comunicación se agrupan bajo el nombre genérico de moléculas de adhesión . La exquisita regulación tanto de la expresión como de la función de estas moléculas dependiendo de cambios en el microambiente celular, no hace difícil imaginar su trascendencia en procesos como la inflamación crónica.

La Artritis Reumatoide constituye, en la investigación biomédica, uno de los paradigmas de enfermedad inflamatoria crónica de base autoinmune. El conocimiento, extraordinariamente rápido, de la presencia de muchas de estas moléculas de adhesión en la membrana sinovial reumatoide está aportando nuevas perspectivas acerca de los mecanismos por los que se puede llegar a la destrucción articular. Las integrinas constituyen un subgrupo de moléculas de adhesión ampliamente representado en los componentes celulares del tejido sinovial , y con especial relevancia funcional en la población mayoritaria de linfocitos T. La estructura general de estos receptores con una porción extracelular, porción intracitoplasmática y región transmembrana permite la transmisión de señales tanto del interior celular hacia afuera, como la transducción de señales hacia el citoplasma desde el exterior. Esta capacidad de conectar el medio extracelular con el intracelular les confiere un especial protagonismo en la regulación de muchos procesos que, como veremos, son esenciales en la patogenia de la enfermedad.

Desde esta perspectiva, podemos imaginar a la membrana sinovial como un gigantesco escenario en el que se suceden múltiples interacciones célula-célula y con la matriz extracelular, orquestadas por mediadores bien conocidos como citoquinas, factores quimiotácticos y activadores celulares. Esta tesis se centra en el estudio de las integrinas, fundamentalmente de la familia β1, en las interacciones secuenciales de linfocitos T con endotelio, matriz extracelular, y fibroblastos sinoviales, y aborda asimismo los posibles mecanismos de regulación.

1. LA MEMBRANA SINOVIAL EN LA ARTRITIS REUMATOIDE

La Artritis Reumatoide (AR) es una enfermedad compleja de origen desconocido en la que factores genéticos, hormonales e inmunológicos interactúan para producir manifestaciones inflamatorias sistémicas y articulares. Se caracteriza por afectar primordialmente a las articulaciones diartrodiales, y suele comenzar como una sinovitis de pequeñas articulaciones de manos y pies que progresa de forma centrípeta, simétrica y deformante. La inflamación articular suele cursar de forma crónica , destructiva y conduce a grados variables de incapacidad.

La respuesta inflamatoria se considera habitualmente como un fenómeno de adaptación biológica que pretende proteger al huésped de factores ambientales hostiles. Un requisito indispensable para que esta respuesta sea *defensiva* es que se lleve a cabo sin dañar significativamente los propios tejidos del huésped. Por lo tanto la respuesta inflamatoria en la AR debe ser considerada como inapropiada. Uno de los mayores interrogantes sigue siendo cómo y por qué las respuestas inmunes aparentemente normales en el estadío agudo progresan a inflamación crónica en los siguientes meses y años.Tanto la rama humoral como la celular de la respuesta inmune parecen participar (Zvaifler 1989). La primera es responsable de la fase exudativa de la artritis. Los anticuerpos producidos localmente en el espacio articular, particularmente los factores reumatoides (FR) [inmunoglobulinas (Ig) anti-Ig M de la articulación] forman complejos con los antígenos y activan el sistema del complemento. La inflamación articular resultante está mediada fundamentalmente por leucocitos polimorfonucleares y sus productos. La inmunidad celular está representada por células inflamatorias crónicas (linfocitos y macrófagos) que infiltran la sinovial y producen factores solubles que perpetúan la inflamación e inducen destrucción tisular (Firenstein et al., 1987, Lanchbury et al., 1993). Para una comprensión adecuada de los mecanismos patogénicos de la enfermedad es importante la apreciación de los cambios que produce la enfermedad en la arquitectura normal de la articulación.

1.1. Membrana sinovial normal y sus componentes.

La articulación diartrodial normal es una estructura funcional contenida dentro de una densa cápsula fibrosa que engloba las dos superficies articulares. El espacio sinovial está delimitado por la membrana sinovial (MS) que carece de lámina basal, y está formada por una capa íntima o superficial (habitualmente designada con el término anglosajón *lining*) y un tejido subintimal, subsinovial o profundo. La capa íntima tiene de 1 a 3 hileras de células diferenciadas del tejido conectivo (sinoviocitos), mientras que el tejido profundo, acelular, contiene en su estroma abundantes capilares sanguíneos. Por su morfología y función se distinguen dos clases de sinoviocitos. Los sinoviocitos "tipo A" son fagocitos, tienen las características fenotípicas de las células histiocíticas derivadas del linaje monocito/macrófago y se renuevan constantemente a partir de precursores de médula ósea. Los sinoviocitos "tipo B'" tienen características fibroblásticas y su origen está menos claro.

1.2. Histopatología de la membrana sinovial inflamada

Los cambios más tempranos en la AR son difíciles de documentar, pero las evidencias disponibles en los días a semanas siguientes a los primeros síntomas articulares (Schumacher et al., 1972) describen el daño endotelial de la microvasculatura. Casi de forma simultánea, se observa infiltración dispersa del espacio subsinovial edematoso por células inflamatorias agudas (polimorfonucleares) y se producen depósitos de fibrina a lo largo de la sinovial. Puede observarse un incremento moderado de las células del *lining* sinovial aunque los linfocitos y células plasmáticas, tan abundantes en la enfermedad establecida, están prácticamente ausentes. En las semanas siguientes, se observa el desarrollo de una extensa red de nuevos capilares, hay obliteración segmentaria de la microvasculatura por células inflamatorias con formación de trombos, y se hace evidente la hiperplasia del *lining* y la acumulación perivascular de linfocitos.

En contraste con las lesiones tempranas, existen múltiples y exhaustivas descripciones de las características histopatológicas de la AR establecida así como de los fenómenos

inmunológicos que perpetúan la reacción inflamatoria inicial (Ishikawa et al.,1976; Young et al., 1984; Zvaifler 1988; Harris, 1990; Panayi 1993; Humbría et al 1992). A medida que la enfermedad se va cronificando se observa un gran incremento en el número y tamaño de las células del *lining* que producen edema y engrosamiento de la sinovial que protruye en la cavidad articular con delgadas vellosidades. El rasgo característico de estas células mesenquimales es su estado de intensa activación con producción de enzimas proteolíticos y prostaglandinas (Díaz González y Alvaro-Gracia, 1992). En el estroma subsinovial se observa infiltración masiva por linfocitos y células plasmáticas, con frecuencia formando pseudofolículos linfoides en particular alrededor de pequeños vasos. Las áreas centradas por vasos muestran, alrededor de ellos, infiltrados en manguito con linfocitos T $CD4^+$ de fenotipo cooperador y que expresan marcadores de activación (Pitzalis et al.,1987; Cush et al., 1988). La mayoría de estos linfocitos exhiben además un fenotipo característico de las células T "de memoria" (Pitzalis et al., 1987; Hanly et al., 1990). La división de las células T en "vírgenes" y "de memoria" representa distintos estados de maduración en la diferenciación de las células T en la periferia: las "vírgenes" no han sido activadas y las "de memoria" son células previamente activadas y que tienden a migrar preferencialmente a los lugares en que han sido estimuladas (Shimizu et al., 1992). Las áreas de transición reclutan macrófagos, linfocitos $CD8^+$ de predominio citotóxico, células plasmáticas y células blásticas. En los centros reactivos linfoides se localizan, en principio, los linfocitos B. El rasgo característico de la AR es la formación de un tejido de granulación agresivo (*pannus*) compuesto de macrófagos y fibroblastos activados que erosiona cartílago, hueso, tendones y ligamentos.

1.3. Mecanismos patogénicos en la Artritis reumatoide

La teoría más aceptada en la actualidad respecto a la etiopatogenia de la Artritis reumatoide se refiere a la presentación por células inmunocompetentes, de un antígeno o antígenos desconocidos a un huésped susceptible para el padecimiento de la enfermedad (Harris 1990, Panayi 1993, Lanchbury 1993). Aunque el antígeno iniciador se desconoce, existe una tendencia creciente a considerar un agente infeccioso como inicial reponsable del estímulo inflamatorio. La continuación

de la respuesta inmune, aún en el caso de que el agente infeccioso hubiera sido eliminado, podría ser perpetuada por antígenos presentes en la propia articulación (colágeno tipo II, proteoglicanos, proteínas de membrana de condrocitos, etc) al ser erróneamente reconocidos como heterólogos y dar así lugar a una respuesta autoinmune. De cualquier modo, el estímulo inmune derivaría de la presentación de antígeno por los macrófagos, en conjunción con moléculas HLA de clase II, a los linfocitos T. Como consecuencia de la estimulación de la célula T, se activarían los linfocitos B para producir anticuerpos, entre otros los FR. Las células T activadas liberarían citoquinas como interferon γ (IFNγ) y otros factores desconocidos que conjuntamente activarían a las células monocito/macrófagicas para liberar una serie de citoquinas como interleuquina-1 (IL-1) y Factor de necrosis tumoral α (TNF-α) A su vez, estas citoquinas, podrían estimular la producción por macrófagos y fibroblastos de otros mediadores inflamatorios como el factor estimulador de colonias granulocítico-macrofágicas (GM-CSF) y factores de crecimiento (TGFβ). Tanto la localización de citoquinas en la sinovial reumatoide como su participación en la fase efectora de la enfermedad han sido revisados exhaustivamente por varios autores (Arend y Dayer, 1990, Alvaro-Gracia 1992, Feldmann et al. 1992, Firenstein 1992, Duff 1993). El análisis tanto de proteínas como de RNA mensajero en el tejido sinovial muestra que los niveles de IL-2, IL-4 e IFNγ (citoquinas típicamente linfocíticas) es bajo o indetectable mientras que las citoquinas de origen monocito/macrófago, especialmente TNFα e IL-1β se encuentran en grandes cantidades. Sin embargo, recientemente, se ha descrito un nuevo procedimiento de inmunohistoquímica que permite la tinción intratisular de citoquinas combinando técnicas de fijación y permeabilización, lo que permite detectar preferencialmente la síntesis local de citoquinas (Andersson y Andersson, 1994). Estos autores consiguen demostrar una presencia sustancial de citoquinas derivadas de células T como IL-2, IL-4, IL-5, IL-10, IL-13, TNFβ e IFNγ, a nivel de proteína, en tejidos sinoviales con inflamación activa obtenidos de pacientes con AR, corroborando el origen en células T mediante una doble tinción con AcM anti-CD3 (Ulfgren et al, 1995).

TNFα induce resorción de cartílago y hueso, estimula la producción de prostanoides y enzimas destructivas por las células sinoviales y aumenta la expresión de antígenos de clase I.

Muchas de estas propiedades son compartidas por IL-1. Así, IL-1 induce a sinoviocitos, condrocitos y fibroblastos a producir enzimas que degradan la matriz extracelular (MEC) como colagenasa, estromelisina, elastasa y activador del plasminógeno que participan en la destrucción del cartílago. GM-CSF estimula macrófagos y aumenta la adhesión de granulocitos al cartílago y su capacidad para degradar proteoglicanos mediante la liberación de radicales libres de oxígeno y enzimas proteolíticos . La figura 1 resume las relaciones intercelulares en la membrana y líquido sinovial con los mecanismos que concurren en la destrucción articular.

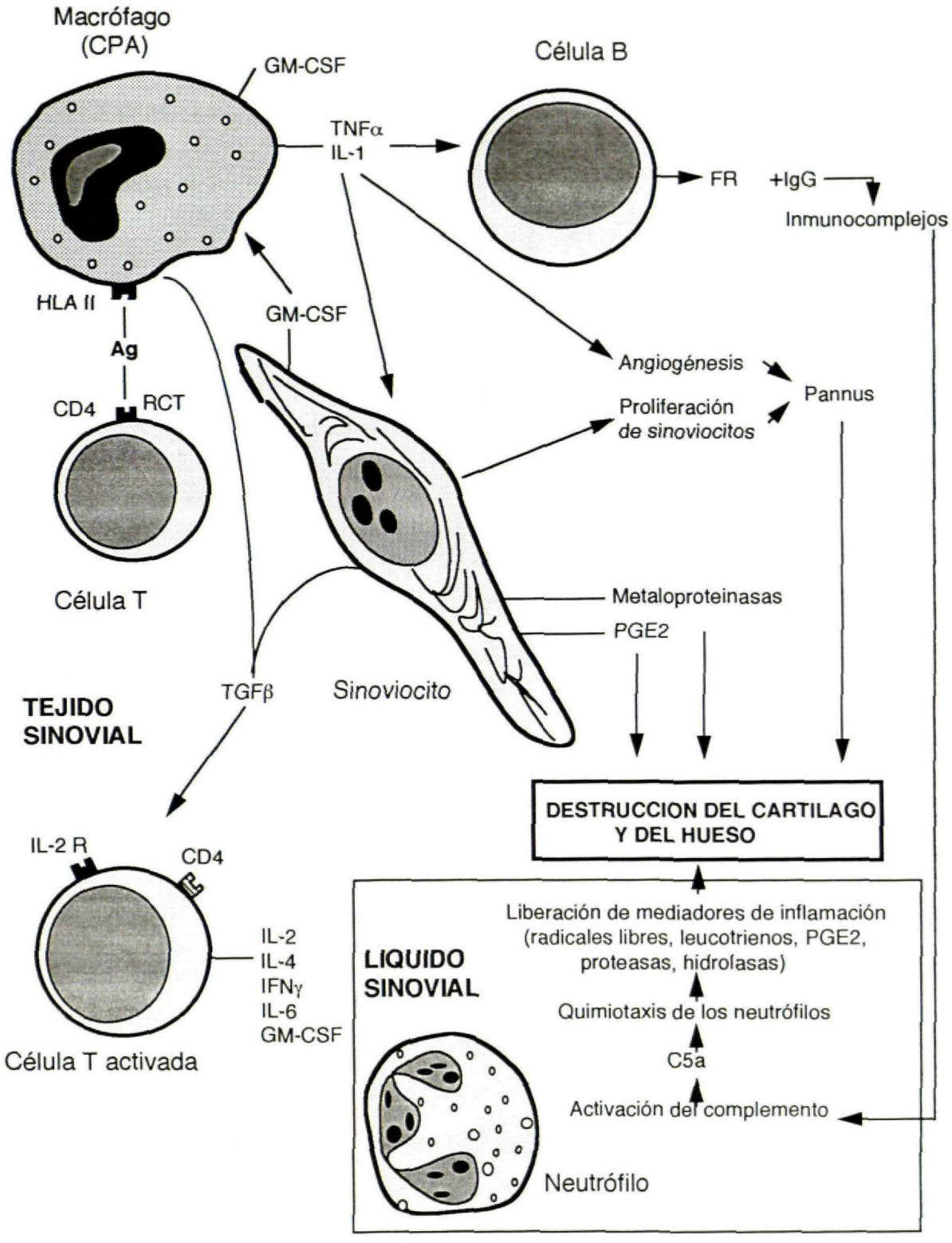

Fig.1. En la Artritis reumatoide, las relaciones intercelulares desencadenadas por el estímulo antigénico, moduladas por múltiples citoquinas y mediadores inflamatorios, desembocan en la destrucción del cartílago y hueso.

2. MATRIZ EXTRACELULAR

Las células en los tejidos producen y secretan una gran diversidad de macromoléculas que forman un complejo entramado que "rellena" los espacios extracelulares dentro de un tejido. Las proteínas que componen la MEC unidas a carbohidratos, habitualmente de forma covalente, se clasifican en glicoproteínas estructurales, proteoglicanos y colágenos (Trelstad 1989). Estas proteínas son las principales especies moleculares responsables de la estructura y función de los tejidos en articulaciones, huesos, cartílagos, tendones, pulmones, piel y córnea.

Algunas de estas proteínas son capaces de funcionar como ligandos, bien en fase sólida o soluble, como receptores y como mediadores de la regulación celular. Por lo tanto, la MEC no sólo sirve como soporte mecánico para mantener la estructura del tejido, sino que modula multitud de funciones celulares como la adhesión, morfología, organización del citoesqueleto, diferenciación, migración y proliferación (Toole et al., 1984; Bernfield et al.,1884; Humphries y Yamada, 1986; Trelstad 1989; Hynes 1990; Juliano y Haskill, 1993). Las proteínas de MEC poseen múltiples dominios de adhesión que median la unión a células y a otros componentes de la MEC. La eventual localización de componentes de la MEC en la superficie de las células viene determinada, en parte, por la presencia de receptores específicos en la membrana celular (Buck y Horwitz, 1987). Una vez unidas, estas moléculas polivalentes pueden unir otros ligandos y amplificar la unión entre la superficie de la célula y el espacio extracelular. Al mismo tiempo, las porciones intracelulares de sus receptores interaccionan con estructuras citoplásmicas, proporcionando una unión física y funcional con eventos intracelulares. Algunos proteoglicanos son componentes integrales de la membrana celular y actúan ellos mismos como receptores (Trelstad 1989). Las glicoproteínas mejor caracterizadas y más ampliamente estudiadas en sus interacciones con células son laminina (LM) (Beck et al., 1990), colágeno (COL) (Tanzer 1989) y fundamentalmente **fibronectina** (FN) (Hynes y Yamada 1982; Yamada 1989; Hynes 1990; Shimizu y Shaw 1991). LM es una gran glicoproteína tripeptídica localizada fundamentalmente en membranas basales, que constituyen una MEC especializada que separa las células endoteliales y epiteliales del tejido subyacente (Timpl

1989). COL engloba una familia de glicoproteínas relacionadas que forman extensas fibras y constituyen uno de los mayores componentes de la matriz intersticial y de las membranas basales.

2.1. Fibronectina: estructura

FN es una glicoproteína dimérica de alto peso molecular que puede encontrarse en forma soluble (plasma y fluídos corporales), o formando agregados en la superficie de las células y en la MEC tisular. Está formada por 2 subunidades, A y B, unidas por puentes disulfuro, cuya estructura primaria consiste en secuencias de aminoácidos repetidas de tres tipos diferentes (I, II, y III) (Hynes 1990). Cada subunidad está compuesta por varios dominios globulares que agrupan subdominios de secuencias repetitivas ordenados linealmente. Estos dominios pueden ser liberados por la acción de proteasas y se corresponden con diferentes dominios funcionales que median la unión a células, COL, heparina y fibrina (Yamada 1989). La figura 2 representa un modelo de la FN plasmática con los diferentes dominios funcionales y los fragmentos resultantes de la digestión proteolítica que contienen las secuencias de unión a células. La región de FN que media la adhesión para la mayoría de los tipos celulares se localiza en parte central de la molécula y se ha identificado como la secuencia Arg-Gly-Asp (RGD), presente en muchas proteínas de MEC y de plasma (Ruoslahti y Pierschbacher, 1986). Los receptores celulares que reconocen esta secuencia, presente en un fragmento proteolítico de 80 kD de la FN plasmática (Fig 2), pertenecen a una familia de MA denominada **integrinas**, y la mayoría se incluyen en la subfamilia **VLA** (Ver epigrafe 3.3 de Introducción)

Aunque la FN se sintetiza a partir de un único gen, las diferentes posibilidades de cortes y empalmes (*splicing* alternativo) que puede sufrir un RNA de transcripción primario genera diversidad de fragmentos polipeptídicos de fibronectina (Schwarzbauer et al., 1983; Kornblihtt et al., 1984). Así, la FN plasmática carece de una de las secuencias repetitivas de tipo III (IIICS) en la subunidad B , mientras que en la FN celular está presente en ambas subunidades (Kornbliht et al., 1984). Este ensamblaje alternativo del RNA origina un lugar de unión a células diferente a la secuencia RGD, que consta de 5 aminoácidos (EILDV) en la región CS-1 (Yamada 1991a). Esta

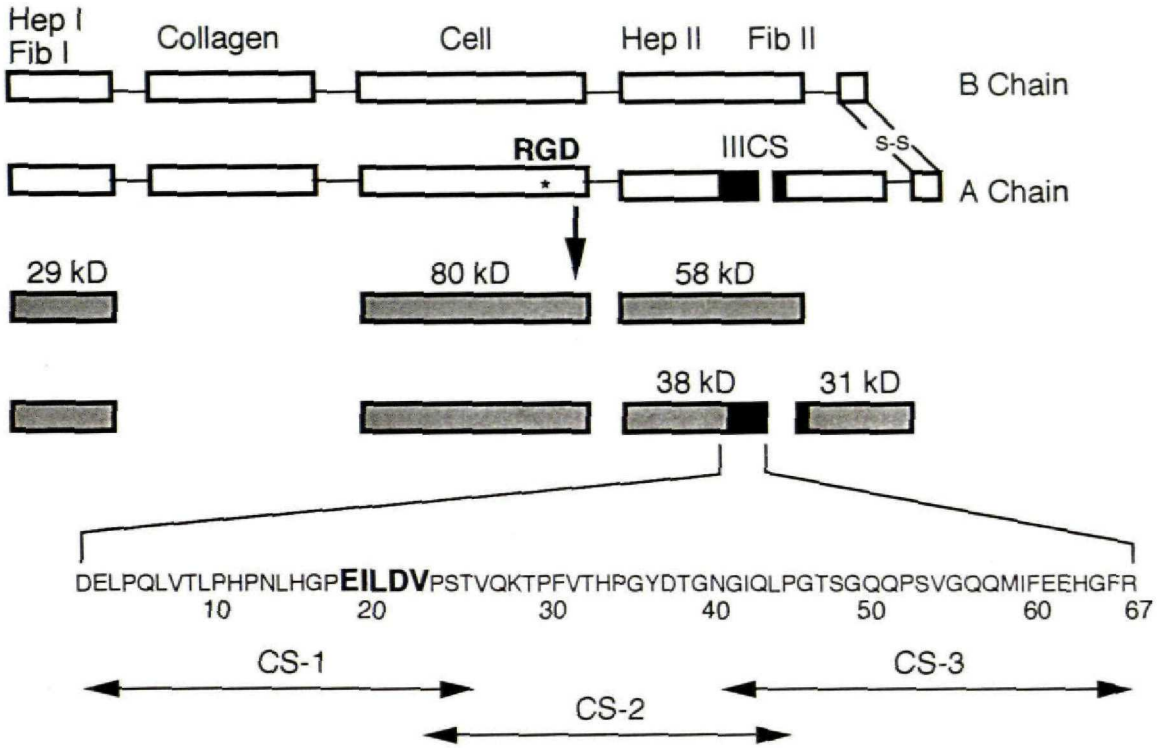

Fig. 2. Esquema de la *fibronectina humana plasmática* que muestra la localización de los dominios biológicamente activos (modificada de García Pardo et al., 1990). Los campos sombreados corresponden a los fragmentos obtenidos por digestión proteolítica. La secuencia RGD, ligando de VLA-5, se encuentra en la parte central de la molécula en el dominio de unión a células, y está contenida en el péptido de 80kD. Las áreas negras IIICS señalan la región derivada de ensamblaje alternativo presente sólo en la cadena A de la FN. El fragmento de 38 kD contiene los primeros 67 aminoácidos de la región IIICS: en el péptido CS-1 se encuentra la secuencia EILDV reconocida por VLA-4.

secuencia está contenida en un fragmento proteolítico de 38 KD que incluye parte del segmento IIICS (Fig 2) y su receptor VLA-4, presente en linfocitos (Wayner et al., 1989; García-Pardo et al., 1990), difiere de otras moléculas VLA que reconocen RGD (Takada et al., 1987). Este procesamiento alternativo viene regulado específicamente por el tipo celular (Kornblihtt et al., 1984) por lo que la acumulación y exposición de diferentes lugares de unión de FN en un tejido puede seleccionar la adhesión de las células que porten el receptor adecuado.

2.2. Fibronectina: síntesis y regulación

Numerosas estirpes celulares sintetizan FN *in vitro* incluyendo fibroblastos, células endoteliales (CE), linfocitos T, macrófagos y polimorfonucleares, y diversos mediadores de inflamación como citoquinas, factores de crecimiento, factores de coagulación además de proteínas de MEC regulan su síntesis (Hynes y Yamada 1982; Nathan y Sporn 1991, Ortiz y Egido 1992). En este contexto se ha visto que células inflamatorias como neutrófilos y macrófagos sintetizan y liberan FN cuando participan en el fenómeno inflamatorio, pero no en condiciones basales (Beaulieu et al.,1985; Menard et al, 1985; Alitalo et al., 1980; Yamauchi et al, 1987). Se han encontrado niveles de FN aumentados en el LS de pacientes con AR (Clemmensen y Andersen, 1982; Kay et al., 1991) lo que parece traducir un aumento de la síntesis local en la articulación. Tanto los fibroblastos como los macrófagos sintetizan FN, por lo que los sinoviocitos tipo A y tipo B son buenos candidatos para esta síntesis. Estudios immunohistológicos indican la presencia del fragmento CS-1 expresado en los vasos y el *lining* de la MS, selectivamente en pacientes con AR, pero no en sujetos controles (Elices et al., 1994). La localización de este fragmento en la superficie luminal de las CE podría favorecer la adhesión y reclutamiento de linfocitos que portaran receptores VLA-4 funcionalmente activos. La presencia de otras formas de FN en la MEC de la MS reumatoide facilitaría el anclaje de células en el tejido por otros receptores VLA adicionales.

3. INTERACCIONES CELULARES Y CON LA MATRIZ EXTRACELULAR: MOLECULAS DE ADHESION.

La adhesión celular desempeña un papel central en muchos procesos biológicos como la morfogénesis, la migración celular y la cooperación célula-célula, con consecuencias directas en fenómenos clínicos tan relevantes como la trombosis vascular, la cicatrización de las heridas, el proceso inflamatorio o las metástasis tumorales. Las **moléculas de adhesión** (MA) son aquellos receptores celulares de superficie que sirven de soporte directo en la unión de las células a su ambiente, ya sea la MEC u otra célula.

La respuesta inmune, tanto su inducción y regulación, como su fase efectora requieren fenómenos de adhesión celular. Los mecanismos que regulan la adhesión y su importancia funcional están particularmente bien representados en el comportamiento de las MA del sistema inmune (Springer 1990; Springer 1994). La transición rápida de una célula desde un estado no adherente (circulante) a uno adherente (migración a un tejido) es fundamental para la función dual del sistema inmune tanto de vigilancia como de respuesta a un estímulo antigénico.

Cuatro fenómenos son esenciales para comprender la función de las MA: a) algunas poseen la capacidad de incrementar reversiblemente la afinidad por su ligando; b) la expresión de los receptores celulares es variable en diferentes tipos celulares, y dentro de estos puede variar según el tejido; c) la expresión de algunas de ellas varía según el estado de activación o diferenciación de la célula, y d) al interaccionar con su ligando son capaces de transmitir señales que controlan, entre otros procesos, la activación y proliferación celular.

La caracterización bioquímica y el clonaje de genes de muchas de estas MA ha permitido su agrupación en 3 familias fundamentales: **selectinas, superfamilia de inmunoglobulinas** e **integrinas**. La figura 3 ilustra, de forma esquemática, la estructura de las moléculas pertenecientes a las diferentes familias. Para evitar descripciones tediosas, nos referiremos en cada grupo a las moléculas implicadas en los procesos que van a ser estudiados en esta tesis, con alusiones recortadas al resto de los receptores.

Un grupo adicional de MA está compuesto por los receptores de la migración linfocitaria específica (Humbría y Laffón 1991; Picker 1992). En condiciones homeostáticas, como parte de su labor en la vigilancia inmunológica, los linfocitos recirculan continuamente entre la sangre y los tejidos linfáticos extravasándose diferencialmente a través de vénulas postcapilares con un endotelio especializado, llamadas vénulas de endotelio alto (HEV) (Girard y Springer, 1995) .Las HEV expresan proteínas de superficie denominadas **adresinas vasculares** que son capaces de unir selectivamente subtipos de linfocitos circulantes que expresan receptores (***homing* receptors**) complementarios (Picker 1992; Carlos y Harlan 1994). En humanos, estas vénulas postcapilares especializadas se encuentran en todos los órganos linfoides secundarios y son capaces de mediar la extravasación de grandes cantidades de linfocitos. Sin embargo, estructuras vasculares similares a HEV se han observado en tejidos crónicamente inflamados, ausentes en áreas de inflamación aguda. Estos datos, junto con estudios recientes *in vivo e in vitro* , sugieren que el estímulo antigénico mantenido, la liberación de mediadores locales secundaria a la activación de células linfoides, y los componentes de la MEC pueden ser responsables de la inducción y mantenimiento de este endotelio especializado (Girard y Springer, 1995). En la MS de AR, cambios en el endotelio con características de HEV, se han detectado en clara relación con la concentración de linfocitos en infiltrados perivasculares (Freemont et al., 1983). Igualmente, la expresión de marcadores de HEV en estos vasos, como HECA-452, es más pronunciada en asociación con grandes infiltrados linfocitarios (Dinther-Janssen et al., 1990). Es interesante reseñar que sistemas específicos en el reclutamiento de linfocitos, similares a los que funcionan en órganos linfoides se han propuesto en la sinovial inflamada (Jalkanen et al. 1986), y recientemente se ha descrito una nueva MA (VAP-1) que media la unión de linfocitos a HEV de tejidos inflamados incluida la sinovial (Salmi et al 1993). Sin embargo, estudios recientes apoyan la participación secuencial de varias moléculas de adhesión conocidas, como selectinas e integrinas, más que la implicación de un sólo par ligando/receptor en la extravasación linfocitaria a través de HEV (Shimizu et al, 1992; Springer 1994; Girard y Springer, 1995).

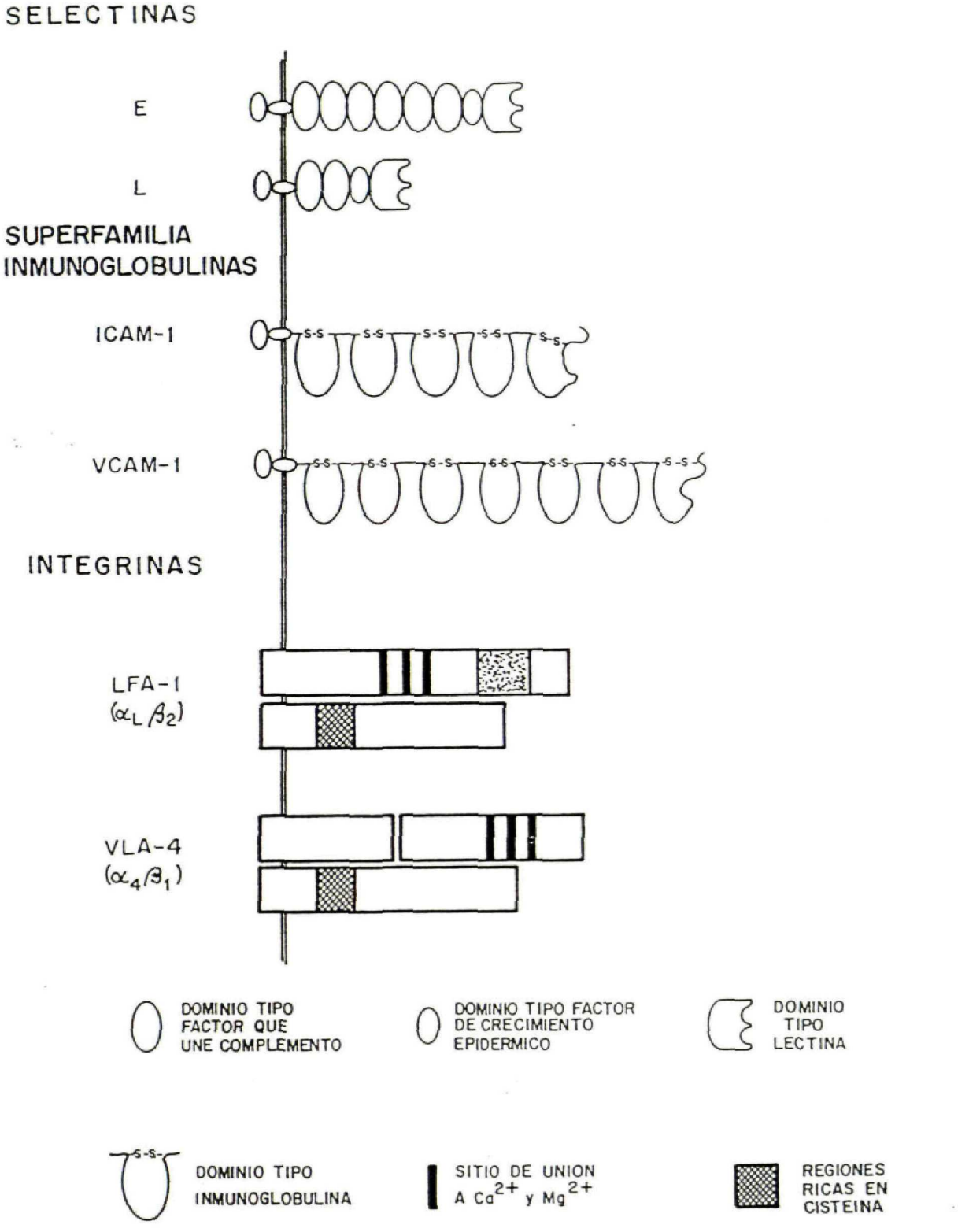

Fig 3. Representación esquemática de la estructura de las moléculas que pertenecen a las diferentes familias de receptores de adhesión.

3.1. Selectinas

La familia de las selectinas comprende 3 glicoproteínas designadas por un prefijo según el tipo celular en el que se definieron inicialmente: selectina E (endotelio), selectina L (leucocito) y selectina P (plaqueta) (Bevilaqua et al,1991). Desde el punto de vista estructural comparten 3 tipos de dominios: dominio NH_2 terminal similar a las lectinas (proteínas que unen carbohidratos) tipo C de mamíferos; dominio similar al factor de crecimiento epidérmico y 2-9 secuencias consenso repetidas, similares a las que aparecen en las proteínas reguladoras del complemento (Bevilaqua y Nelson 1993; Carlos y Harlan, 1994) (Ver Fig. 3). Se caracterizan por su capacidad de unión de oligosacáridos a través de su dominio lectina y por la dependencia de Ca^{++} para poder unir ligandos. Los contrarreceptores o ligandos de las selectinas son determinantes carbohidratados que se ubican en estructuras terminales de grupos azucarados de una o más glucoproteínas o glucolípidos, proporcionando lugares de reconocimiento específico para cada proteína que es capaz de unirse a ellos (Lasky , 1992; Rosen y Bertozzi, 1994)

La **selectina L** (LAM-1, CD62L) se expresa de forma constitutiva en la mayoría de los linfocitos, en polimorfonucleares, monocitos y otras células mieloides (Tedder et al, 1990), aunque el peso molecular en los diferentes tipos celulares varía por diferencias de glicosilación postranslacionales. Aunque se definió inicialmente como un receptor de migración específico de linfocitos murinos a ganglios linfáticos (Gallatin, 1983), esta selectina media la extravasación de neutrófilos a lugares de inflamación, específicamente en la adhesión a endotelio activado (Hallman et al., 1991). Estudios realizados *in vivo* , mediante microscopía intravital, indican que la selectina L media la primera interacción de los neutrófilos con el endotelio, frenando su velocidad y permitiendo a los leucocitos circulantes rodar sobre el endotelio (fenómeno conocido como *rolling*) (von Adrian et al, 1991; Ley et al, 1991) antes de que se establezca la unión firme y extravasación. Igualmente, en condiciones de flujo, los monocitos realizan *rolling* sobre endotelio estimulado con IL-4 a través de la interacción de la selectina L con su ligando (o ligandos inducibles) (Luscinskas et al. 1994). Los leucocitos pierden la selectina L durante su activación, liberándose al medio por un mecanismo proteolítico, que constituye así un mecanismo de regulación de su capacidad adhesiva y

migratoria (Spertini et al, 1991). Se han caracterizado 3 ligandos biológicos de la selectina L que comparten una organización tipo mucina : GlyCAM-1/sgp50 , MAdCAM-1 y CD 34/sgp90 (Rosen y Bertozzi, 1994). Gly-CAM y CD34 son ligandos asociados a HEV para la selectina L (Lasky et al 1992; Baumhueter et al 1993). MAd-CAM-1 en las HEV de placas de Peyer intestinales es el ligando vascular de la integrina linfocitaria $\alpha 4/\beta 7$ y es capaz de mediar el *rolling* de linfocitos a través de esta integrina (Berlin et al, 1995). Además, el subgrupo de Mad-CAM-1 presente en las HEV de gaglios linfáticos intestinales soporta el *rolling* de linfocitos mediado por la selectina L (Berg et al., 1993).

La **selectina P** (CD62P, GMP 140) se encuentra almacenada en gránulos en las CE y plaquetas, translocándose rápidamente a la membrana tras la activación celular, donde es capaz de unirse a sus ligandos, pero se internaliza poco después (McEver et al., 1989). La selectina P participa en la unión de plaquetas activadas a neutrófilos y monocitos y, como la selectina L, interviene en el *rolling* de neutrófilos al endotelio inflamado (Carlos y Harlan 1994). El ligando en neutrófilos para esta selectina , PSGL-1, también es una proteína con un gran dominio mucina (Rosen y Bertozzi, 1994). Recientemente se ha descrito la capacidad de selectina P para mediar la unión inicial de linfocitos T CD4+ a HUVEC (Células endoteliales de cordón umbilical humano) estimuladas con TNFa, en condiciones de flujo (Luscinskas et al, 1995). Estudios inmunohistoquímicos han demostrado la presencia de la selectina P en el endotelio de tejidos sinoviales, pero su expresión no está aumentada en la MS de AR respecto a la de artrosis o sujetos sanos (Johnson et al., 1993), por lo que su papel funcional *in vivo* está por definir.

La expresión de **selectina E**, también llamada ELAM-1 o CD62E, está restringida a CE activadas *in vitro* o *in vivo* y requiere síntesis activa de proteínas. Se expresa en un plazo de horas en CE activadas por citoquinas como IL-1 y TNFα y, activadores como linfotoxina, endotoxina bacteriana o el neuropéptido sustancia P (Bevilaqua et al.,1989). ELAM-1, con nula o muy reducida expresión en HEV de tejidos linfoides secundarios, se ha encontrado en vénulas de lesiones inflamatorias crónicas del tracto gastrointestinal, pulmón, tracto genitourinario, tiroides, entre otros, pero expresándose con especial intensidad en enfermedades cutáneas y sinoviales

crónicas (Picker et al.,1991). En cortes histológicos de tejido sinovial de AR, ELAM-1 se expresa en CE con mayor frecuencia que la encontrada en artrosis (Koch et al., 1991). Técnicas combinadas de hibridación *in situ* e inmunohistoquímica han demostrado que la presencia de RNA mensajero y de la proteína ELAM-1 en CE de MS se correlaciona con la actividad inflamatoria de la AR (Kriegsmann et al, 1995). De especial interés son los ensayos realizados con el aislamiento y cultivo de CE procedentes de la microvasculatura de la MS de AR. En respuesta a IL-1, se ha demostrado una mayor inducción de ELAM-1 en las CE sinoviales respecto a las obtenidas de cordón umbilical (Abbot et al., 1992). En ensayos *in vitro* de adhesión a HUVEC, la selectina E media la adhesión de neutrófilos, monocitos, eosinófilos, basófilos y células de ciertos carcinomas (Kishimoto 1993). Esta selectina media únicamente la adhesión de células T de memoria y su unión es independiente de la activación celular, que regula la adhesión firme mediada por integrinas (Shimizu et al., 1991a; Shimizu et al, 1991b). Aunque no se conocen los ligandos naturales de ELAM-1, excepto la selectina L de neutrófilos, los determinantes carbohidratados que reconoce portan el antígeno llamado sialylLewis X (SLe^X) o azúcares relacionados sialilados o fucosilados (Kishimoto 1993; Rosen y Bertozzi, 1994). Este grupo SLe^X y otros relacionados se expresan en la selectina L de neutrófilos y monocitos y son reconocidos por el anticuerpo monoclonal (AcM) HECA-452, que además de ser un marcador de HEV (Girard y Springer, 1995), define una subpoblación de células T de memoria capaces de unirse a ELAM-1 (Picker et al, 1991). El antígeno reconocido por este AcM, Antígeno Cutáneo Asociado, se expresa en la mayoría de las células T de memoria, tanto circulantes como de la piel , de enfermos con dermatitis crónicas (Picker et al., 1991).

3.2. Superfamilia de las inmunoglobulinas

Los miembros de esta familia comprenden un grupo importante de glicoproteínas de membrana, con función y distribución muy diversa , que se caracterizan por poseer dominios estructurales semejantes a las Ig (Williams y Barclay, 1988) (Fig. 3). Muchas de ellas se expresan en linfocitos e incluyen los receptores para el antígeno de los linfocitos B y T (RCT), el complejo

CD3 asociado a RCT, moléculas HLA de clase I y II, CD4, CD8, CD22 y CD28 (Springer 1990, Laffón 1992). Todas ellas participan en la respuesta y el reconocimiento inmune.

Además, esta superfamilia incluye moléculas de adhesión celular implicadas en la adhesión y activación celular T, incluso antígeno-independiente, (CD2, LFA-3) y varios ligandos endoteliales que participan en la adhesión de leucocitos al endotelio vascular (ICAM-1, ICAM-2, VCAM-1)

La molécula **CD2** posee dos dominios extracelulares de tipo Ig y su expresión está restringida a células T y "natural killer" (NK), pero su principal ligando **LFA-3** (CD58) está presente en la mayoría de los tipos celulares (Selvaraj et al., 1987). Las interacciones mediadas por CD2 están involucradas en la adhesión de la célula T con muchas otras células y su largo fragmento intracelular es capaz de transmitir señales activadoras, que aumentan o son sinérgicas con las transmitidas por el RCT (Bierer et al., 1989). LFA-3 también pertenece a la superfamilia de las Ig y tiene varias isoformas dependiendo de su forma de anclaje a la membrana. Ciertos AcM frente a CD2 y LFA-3 impiden la adhesión macrófago-linfocito T necesaria para el reconocimiento y respuesta al antígeno y, la integridad de la interacción CD2/LFA-3 es critica para estabilizar las interacciones de linfocitos T citotóxicos con su célula diana (Bierer et al., 1990). La activación de la célula T incrementa la adhesión entre CD2 y LFA-3 probablemente en relación con una mayor densidad de receptores CD2, pobremente sialilados y con epítopos conformacionales activos que no están presentes en células T en reposo (Springer 1990). Asimismo, LFA-3 puede funcionar como una de las señales activadoras para CD2 pero se necesita un segundo ligando sinérgico para desencadenar la activación de la célula T (Dustin et al., 1989a; Bierer et al., 1989). En este sentido se ha descrito otro ligando fisiológico para CD2, CD59, aunque se desconoce si puede cooperar con LFA-3 para desencadenar la activación fisiológica de la célula T en ausencia del antígeno. Este fenómeno podría tener relevancia fisiológica en la MS reumatoide, donde se ha detectado la presencia de LFA-3 en sinoviocitos tipo macrofágico, fibroblastos sinoviales, eritrocitos dentro de los vasos, endotelio y en linfocitos infiltrantes, mientras que CD2 sólo se expresa en linfocitos T del infiltrado (Hale et al., 1989).

Los ligandos endoteliales de las moléculas leucocitarias (integrinas) que median adhesion firme a endotelio pertenecen también a la superfamilia de las Ig, aunque algunos de ellos tienen además expresión extravascular y son capaces también de mediar interacciones con otros tipos celulares.

ICAM-1 (CD54) es una glicoproteína monocatenaria con 5 dominios tipo Ig (Fig. 3) expresada constitutivamente por las CE y una gran variedad de células como leucocitos poli y mononucleares, macrófagos y fibroblastos (Dustin et al.,1986). La expresión basal de ICAM-1 en CE puede ser incrementada selectivamente por IFNg, TNFa, IL-1 y lipopolisacárido (LPS) de las paredes bacterianas. (Dustin et al.,1986; Carlos y Harlan 1994). Este incremento se detecta a las 4 horas y, en contraste con la expresión transitoria de selectina E, se mantiene más de 24 h. (Carlos y Harlan, 1994). **ICAM-2** sólo contiene 2 dominios tipo Ig, se expresa constitutivamente en la mayoría de leucocitos y en CE pero su expresión no se modifica por. la acción de citoquinas (Staunton et al., 1989). Ambas moléculas son ligandos de la integrina linfocitaria LFA-1, pero sólo ICAM-1 es capaz de interaccionar con otra integrina de leucocitos denominada Mac-1 (Springer 1990). Existe un tercer ligando para LFA-1 en esta superfamilia, denominado **ICAM-3**, pero a diferéncia de los anteriores está restringido a células de estirpe hematopoyética (Fawcet et al., 1992) y tiene importantes funciones reguladoras en las procesos de adhesion de células T mediados por integrinas (Campanero et al., 1994).

VCAM-1 se describió inicialmente como una molécula inducible en la superficie de CE en cultivo despúes de 2-4 horas tras el estímulo con diversas citoquinas. IL-1, TNFa (Carlos et al., 1990), IL-4 (Masinovsky et al., 1990), y LPS pero no IFNγ (Osborn et al., 1989) inducen la expresión de VCAM-1 en HUVEC. Posteriormente se ha encontrado expresión tanto constitutiva como inducible en otras estirpes celulares, y está presente en vénulas de tejidos inflamados (Postigo et al., 1993). La molécula tiene un número variable de dominios tipo Ig que dependen del ensamblaje ó *splicing* alternativo postranscripcional. La forma predominante en el endotelio tiene 7 dominios (fig. 3), pero se ha descrito una con 3, específica de tejidos inflamados y anclada a la membrana por una unión fosfatidil inositol (Carlos y Harlan, 1994) Media la adhesión firme de

linfocitos, monocitos, eosinófilos y basófilos al endotelio activado a través de la integrina VLA-4 ($\alpha 4\beta 1$) (Postigo et al, 1993; Lobb y Hemler, 1994). Recientemente, ensayos de adhesión tanto *in vitro* como *in vivo*, han demostrado la capacidad de VLA-4 ($\alpha 4\beta 1$) para sustentar el contacto inicial y el rodamiento de linfocitos y eosinófilos sobre VCAM-1 de CE en condiciones de flujo (Jones et al., 1994; Berlin et al.,1995; Luscinskas et al.,1995; Sriramarao et al., 1994). Este hallazgo es de especial relevancia, ya que hasta ahora se pensaba que sólo las selectinas eran capaces de frenar al leucocito circulante e iniciar la adhesión endotelial.

Estudios de inmunofluorescencia en la MS reumatoide, han demostrado la presencia de VCAM-1 en algunas células del *lining* sinovial y sobre todo en macrófagos del intersticio, además de una alta expresión en vasos (Koch et al, 1991). ICAM-1 muestra una distribución más amplia, con presencia en CE de arteriolas y vénulas, algunos macrófagos, fibroblastos y linfocitos del espacio subsinovial y en sinoviocitos de la capa bordeante (Hale et al., 1989; Koch et al., 1991). Adicionalmente, la expresión constitutiva de estas dos moléculas en sinoviocitos tipo fibroblasto en cultivo, se puede incrementar por la acción de IL-1, TNFα e IFNγ, y por IL-4 en el caso de VCAM-1 (Morales-Ducret et al, 1992). Por el contrario la expresión de LFA-3 en estas células no se modifica por citoquinas (Chin et al., 1990).

CD31 es una glicoproteína transmembrana cuya región extracelular se organiza en 6 dominios tipo Ig y es capaz de mediar interacciones homofílicas y heterofílicas (DeLisser et al., 1994) Inicialmente descrita en plaquetas como molécula de adhesión a endotelio (PECAM-1), se expresa constitutivamente en el endotelio de todos los tipos de vasos, donde se concentra en las uniones célula-célula (Carlos y Harlan, 1994). En leucocitos se ha identificado en monocitos, neutrófilos y en subgrupos de linfocitos, particularmente $CD8^+$ vírgenes, asi como en células progenitoras mieloides de médula ósea (Carlos y Harlan, 1994). Estudios recientes apoyan su participación en el reclutamiento de leucocitos a focos inflamatorios, en la migración transendotelial de neutrófilos y monocitos (probablemente mediada por interacciones homofílicas) y en la angiogénesis (DeLisser et al., 1994). El primer ligando heterofílico conocido de CD31, en la adhesión de leucocitos al endotelio, ha sido comunicado recientemente y corresponde a la integrina

αvβ3 (Piali et al., 1995). Asimismo, CD31 es capaz de transmitir señales activadoras en linfocitos T $CD8^+$, aumentando su capacidad adhesiva a través de la activación de integrinas α4 (Tanaka et al, 1992). La expresión de CD31 está aumentada en la MS de AR respecto a la de artrosis; se presenta en > 90% de las CE y su expresión en macrófagos subsinoviales y sinoviocitos del *lining* se correlaciona positivamente con el grado de inflamación de los tejidos estudiados (Johnson et al., 1993).

3.3. Integrinas

Las integrinas son proteínas de superficie con una porción intracitoplásmica, porción transmembrana y otra extracelular capaces de conectar el citoesqueleto con el medio extracelular y generar señales en ambos sentidos (Hynes 1992). Desde el punto de vista estructural son heterodímeros compuestos por 2 cadenas polipeptídicas α y β que se asocian de forma no covalente y son transportadas a la superficie celular como un complejo. Hasta el momento 8 cadenas b conocidas y 12 de las 15 a descritas han sido clonadas (Hynes 1992, Smyth et al.,1993). Las integrinas se clasifican en subfamilias de acuerdo con una cadena β común a la que se unen diferentes cadenas α , aunque algunas subunidades a pueden asociarse con diferentes cadenas β (Fig. 4A). De este modo con 22 combinaciones diferentes de asociaciones αβ se convierten en el grupo más numeroso de MA conocido en humanos, cuya estructura y relevancia funcional se ha recogido en múltiples revisiones de la literatura reciente (Ruoslahti 1991; Hynes 1992; Sánchez-Madrid y Corbí 1992; Ginsberg et al, 1992; Smyth et al., 1993; Lucinskas y Lawer 1994; Sánchez-Madrid y González-Amaro, 1995). La distribución tisular de las integrinas es muy amplia y prácticamente todos los tipos celulares expresan varios complejos αβ. Mediante la síntesis de determinadas cadenas α y β la célula puede seleccionar el reconocimiento de ligandos específicos. El sitio de unión al ligando está formado por regiones de las cadenas α y β, y la interacción receptor-ligando depende habitualmente de la presencia de Ca^{++} y Mg^{++}.

Todas las subunidades a presentan una característica común, que es la presencia en su región extracelular de 3-4 dominios de unión de cationes divalentes, de gran importancia tanto para

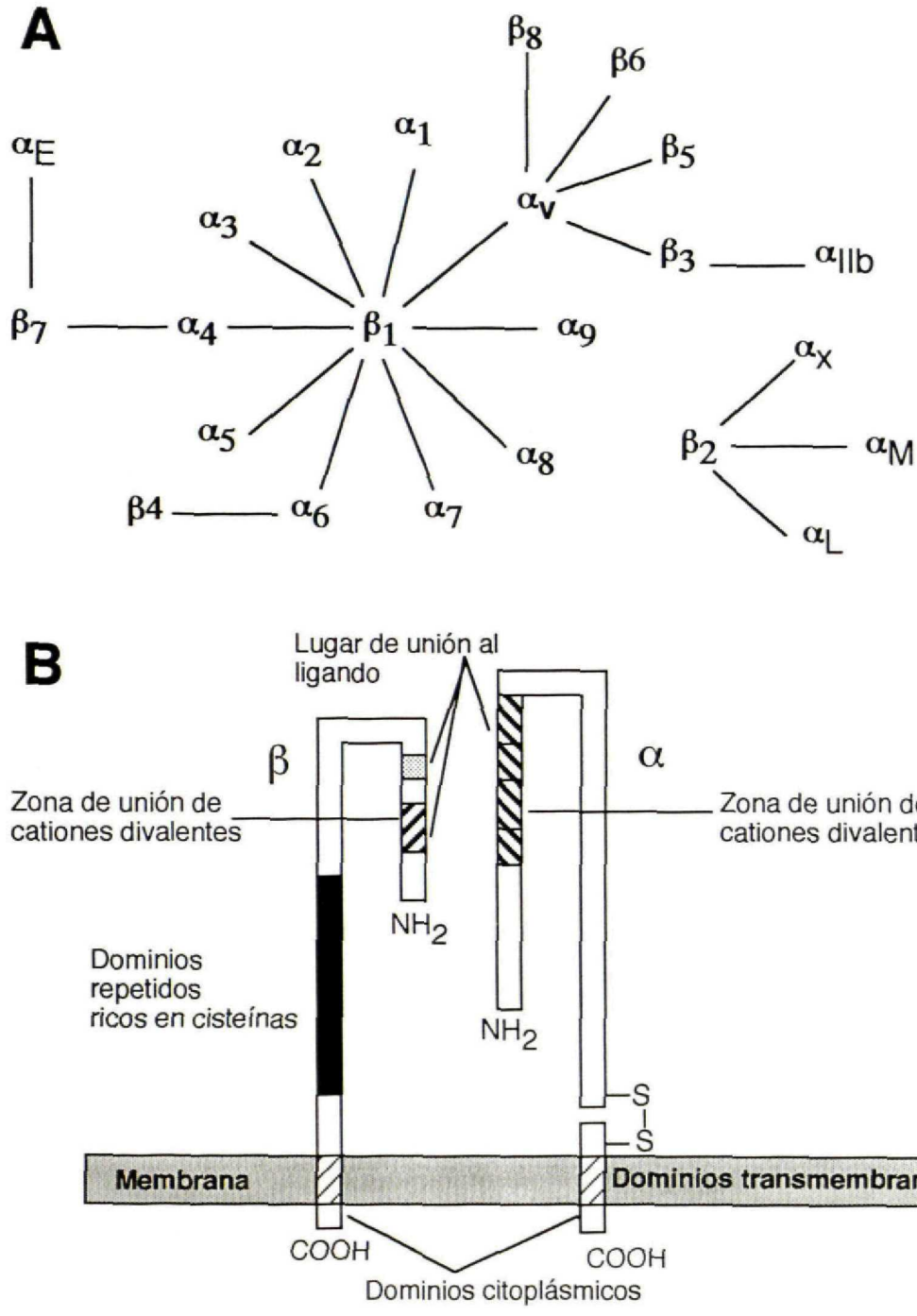

Fig.4. Representación esquemática de las diferentes asociaciones αβ (A) y de la estructura general de una integrina (B). Se representa la localización de los diferentes dominios estructurales. Los lugares de unión al ligando han podido ser identificados mediante estudios químicos de entrecruzamiento y mapeando la pérdida de función inducida por mutaciones. No todas las subunidades α son separadas proteolíticamente en dos subunidades unidas por puentes disulfuro, y algunas contienen sólo tres regiones de unión de cationes divalentes.

la interacción con la subunidad β como para la unión de los ligandos. Algunas de ellas son escindidas postraduccionalmente en 2-3 péptidos que permanecen unidos por puentes disulfuro. Las cadenas α que no sufren esta escisión proteolítica contienen una región extracitoplasmática adicional denominada dominio I. Por su parte, la subunidad β contiene 5 dominios repetidos ricos en cisteínas situados en una zona próxima a la región transmembrana, y todas muestran una gran homología de secuencia en la mitad amino-terminal de la molécula, que parece una región implicada en la interacción con los ligandos. Las cadenas β poseen, además, dominios de unión a cationes divalentes que, como ocurre en las cadenas α , están muy cerca de los lugares de unión al ligando. La región citoplasmática de la cadena β interacciona con componentes del citoesqueleto y puede estar implicada en la generación de señales intracelulares. Tanto en las cadenas α como en las β opera un mecanismo de generación de diversidad molecular mediante un procesamiento alternativo de sus RNAm que da origen a isoformas con dominios citoplasmáticos distintos. La estructura esquemática de una integrina se representa en la figura 4B.

El hecho de que una subunidad β pueda asociarse a subunidades α diferentes ha permitido clasificar a las integrinas en varias subfamilias: a) β1, que comprende el heterodímero αvβ1 y las moléculas VLA (*very late activation antigens*) con nueve subunidades α diferentes (α1-α9), b) β2 o integrinas leucocitarias con tres subunidades α diferentes(αL, αM y αX) c) β3 o citoadhesinas con las cadenas αIIb y αv y d) β7, de expresion restringida a leucocitos que se asocia a α4 y αE. De estas 4 familias, varios miembros de la familia β1 y β2 presentes en linfocitos T median interacciones con el endotelio activado, con la proteína de MEC fibronectina y con sinoviocitos tipo fibroblasto.

3.3.1. Subfamilia VLA (β1)

Las integrinas VLA comparten la molécula CD29 como subunidad b común. Su denominación se debe a la descripción inicial de VLA-1 y VLA-2 como antígenos de activación tardía de linfocitos T (Hemler et al., 1984), pero su identidad como grupo viene dada por constituir un conjunto de receptores celulares para proteínas de MEC como FN, COL, LM y vitronectina

(VN) (Tabla 1). VLA-4 es capaz de mediar, además, interacciones célula-célula (Hemler et al., 1990). Con excepción de los neutrófilos, todas las células del organismo expresan en su membrana una o más integrinas β1 (De Estrooper et al., 1989; Hemler 1990).

3.3.1.a. Distribución

La distribución de las integrinas VLA en leucocitos se ha descrito ampliamente en revisiones recientes (Hemler 1990; Springer 1990; Sánchez-Madrid y Corbí 1992). VLA-1 y VLA-2 se expresan en linfocitos T activados y en bajos niveles en monocitos, pero son casi indetectables en linfocitos B y células T en reposo. La expresión de VLA-4 es alta y homogénea en monocitos, timocitos y linfocitos B pero varía en linfocitos T. Su expresión es mayor en células T activadas y células T de memoria respecto a linfocitos T en reposo o vírgenes. VLA-5 se expresa moderadamente en monocitos, es mínima en timocitos y células B en reposo y varía en linfocitos T, pero no aumenta con la activación. VLA-6 está ausente en células B pero se expresa abundantemente en monocitos y moderadamente en linfocitos T y timocitos. En resumen, los linfocitos T y B en reposo expresan VLA-4, niveles discretos pero detectables de VLA-3, y difieren en la expresión de VLA-5 Y VLA-6. Los monocitos tienen una alta expresión de VLA-6 y moderada de VLA-2, VLA-4 y VLA-5. Ninguna integrina VLA se expresa en polimorfonucleares, con la excepción de eosinófilos y basófilos que expresan VLA-4.

3.3.1.b. Función

Receptores de MEC. Todos los miembros de la familia VLA pueden funcionar como receptores de MEC y la mayoría de ellos son capaces de reconocer más de un ligando (Tabla 1). VLA-1 es capaz de unir COL (Kramer y Marks, 1989) y LM (Ignatius y Reichard, 1988). VLA-2 actúa como receptor de COL o receptor de COL/LM dependiendo del tipo celular en el que se exprese (Elices y Hemler, 1989). VLA-3 tiene un amplio espectro de ligandos que incluyen COL, LM , FN (Elices et al., 1991) y epiligrina, una glicoproteína presente en la mayoría de las membranas basales epiteliales (Carter et al, 1991). VLA-6 y VLA-7 median la unión a LM (Gehlsen et al., 1988; Kramer et al., 1991). VLA-5 es bien conocido como el prototipo de receptor

Tabla 1. Integrinas β1 y β2: miembros, distribución celular y ligandos

	Ligandos	Distribución celular
SUBFAMILIA β1		
α1β1 (VLA-1)	COL, LM	Amplia
α2β1 (VLA-2)	COL, LM	Amplia
α3β1 (VLA-3)	COL, LM, FN, Epiligrina	Amplia
α4β1 (VLA-4)	FN (CS-1), VCAM-1, Trombospondina, invasina bacteriana	Leucocitos, melanomas
α5β1 (VLA-5)	FN (RGD), Trombospondina	Amplia
α6β1 (VLA-6)	LM	Plaquetas, granulocitos
α7β1	LM	Melanomas
α8β1	No determinado	Cél.ep., neuronas, melanomas
αvβ1	VN, FN	Cél. ep., neuronas
SUBFAMILIA β2		
αLβ2 (LFA-1, CD11a/CD18)	ICAM-1, ICAM-2, ICAM-3	Leucocitos
αMβ2 (Mac-1, CD11b/CD18)	ICAM-1, FG, C3bi, fX	Células mieloides
αxβ2 (p150/95, CD11c/CD18)	FG, C3bi	Células mieloides, Células B

COL: Colágeno; Cél. Ep: Células epiteliales; FG: Fibrinógeno; FN: Fibronectina; fX: factor X de la coagulación; LM: laminina; VN: vitronectina.

de FN y su ligando es el dominio de unión celular de la FN que contiene la secuencia RGD (Pytela et al., 1985; Wayner et al., 1989). VLA-4 se describió como receptor de FN en linfocitos que, además de utilizar VLA-5, pueden reconocer otra secuencia, derivada de ensamblaje alternativo, en el dominio CS-1 de la FN a través de $\alpha 4\beta 1$ (Wayner et al., 1989).

Adhesion célula-celula. VLA-4 es la integrina $\beta 1$ implicada por excelencia en interacciones célula-célula, lo que le confiere una especial relevancia funcional *in vivo*. Participa en fenómenos de agregación homotípica leucocitaria y en las interacciones de linfocitos T citotóxicos con la célula diana a través de ligandos todavía desconocidos (Takada et al., 1989; Campanero et al., 1990). La interacción con su ligando VCAM-1 está implicada en múltiples procesos fisiológicos y patológicos (Postigo et al., 1993; Lobb y Hemler 1994): 1) media la interacción de linfocitos, monocitos, eosinófilos y basófilos al endotelio inflamado, por lo que participa en fenómenos inflamatorios y alérgicos ; 2) la producción de metástasis por ciertos tumores se relaciona con la expresión de $\alpha 4\beta 1$ y su capacidad de unión a endotelio; 3) media la unión de precursores hematopoyéticos al estroma de médula ósea, por lo que AcM anti $\alpha 4$ son capaces de reclutar a SP células madre (*stem cells*) hematopoyéticas; 3) participa en la interacción de linfocitos T y B con células dendríticas, fenómenos importantes en la estimulación antigénica; 4) regula la diferenciación del músculo esquelético. La redistibución de VLA-2 y VLA-3 en los puntos de contacto intercelular de queratinocitos en cultivo, y la implicación de VLA-2 en la agregación leucocitaria mediada por la cadena $\beta 1$ sugieren la existencia de un ligando celular para estas integrinas (Carter et al., 1990; Campanero et al., 1992).

3.3.2.Subfamilia $\beta 2$

Las integrinas que comparten la cadena $\beta 2$ (CD18) se denominan también integrinas leucocitarias y comprenden tres heterodímeros con expresión restringida a leucocitos (Ver Tabla 1). **LFA-1** (CD11a/CD18) se expresa en todos los tipos de leucocitos y células *natural killer*; **Mac-1** (CD11b/CD18) y **p150/95** (CD11c/CD18) están presentes en células de la serie mieloide y células

natural killer , aunque esta última puede encontrarse en células dendríticas, ciertos clones T citotóxicos y linfocitos B activados. (Sánchez-Madrid y Corbí, 1992).

La función de las integrinas β2 es fundamentalmente la adhesión intercelular. LFA-1 participa en la citotoxicidad mediada por células T y *natural killer* ,en las respuestas proliferativas de linfocitos T y B, en la citotoxicidad celular de monocitos y granulocitos mediada por anticuerpos, y en la adhesión de leucocitos al endotelio (Larson y Springer, 1990). Todas estas funciones están mediadas por la interacción de LFA-1 con sus ligandos en la superfamilia de las Ig, ICAM-1, ICAM-2 e ICAM-3, que han sido descritos previamente. Las interacciones de LFA-1 con ICAM-1 e ICAM-2 en la superficie de las CE facilitan los procesos de extravasación de leucocitos mono y polimorfonucleares. Su interacción con ICAM-1 e ICAM-3 es importante en interacciones entre leucocitos como actividad citotóxica, cooperacción T-T, T-B y con células presentadoras de antígeno. A través de su ligando ICAM-1 puede unirse a células de estirpe no mieloide como fibroblastos.

Al igual que LFA-1, Mac-1 y p150/95 intervienen en la adhesión de monocitos y neutrófilos al endotelio a través de su unión a ICAM-1 y a un ligando desconocido, respectivamente. Mac-1 participa, además, en procesos de agregación y quimiotaxis de neutrófilos y monocitos, y por su capacidad como receptor del complemento está implicado en la fagocitosis (Sánchez-Madrid y Corbí, 1992).

3.3.3. Regulación de la expresión y la función de integrinas β1 y β2

Las células pueden modular sus capacidades adherentes fundamentalmente a través de dos mecanismos: 1) variando los niveles de expresión en el repertorio de integrinas, 2) modulando la especificidad y afinidad de las integrinas por sus ligandos (Hynes, 1992; Pardí et al, 1992; Ginsberg et al, 1992; Smyth et al, 1993).

Las integrinas β2 se expresan constitutivamente en la superficie de células no activadas, y por lo general presentan una conformación funcionalmente inactiva. Mac-1 y p150/95 se

encuentran además almacenadas en depósitos intracelulares (gránulos secundarios y terciarios) dentro de los neutrófilos y monocitos. La activación celular provoca la rápida movilización y fusión de los gránulos intracitoplásmicos con la membrana plasmática, aumentando así su expresión en la superficie celular (Miller et al, 1987). Por otro lado, la activación de leucocitos en respuesta a factores quimiotácticos, citoquinas, antígenos o mitógenos provoca cambios conformacionales en el receptor que le permite unirse de forma eficaz a su ligando sin aumentar el número de receptores (Larson y Springer, 1990). Estos mecanismos de regulación aseguran que los leucocitos permanezcan circulantes en condiciones homeostáticas, disponibles para adquirir propiedades adhesivas sólo cuando sea necesario.

Una situación análoga existe en los linfocitos T. La activación celular conduce a un aumento de expresión de LFA-1 y VLA-4 y es capaz de inducir la expresión de VLA-1 y VLA-2. Por otro lado, la activación comporta cambios cualitativos hacia formas activas de VLA-4, VLA-5 y VLA-6 que incrementan la capacidad de interacción con sus ligandos celulares o proteínas de MEC (Shimizu et al., 1990). Por otra parte, en las células T maduras, la expresión del antígeno CD45R0, que las identifica como células de memoria, se acompaña de un incremento en los niveles de LFA-1, VLA-4, VLA-5, VLA-6, CD2 y LFA-3 (Sanders et al., 1988; Shimizu et al.; 1990). Estas diferencias fenotípicas podrían explicar, al menos en parte, los diferentes patrones de recirculación que exhiben las células T de memoria frente a las vírgenes (Shimizu et al, 1992; Springer 1994). Así las células de memoria migran preferencialmente a tejidos no linfoides, inflamados o no, y tienden a regresar al tejido en el que fueron inicialmente estimuladas.

Como hemos visto, la expresión de una integrina no implica que sea funcionalmente activa. El cambio de afinidad de una integrina por su ligando contituye un mecanismo rápido y versátil de la célula para modificar sus propiedades adhesivas. Desde el interior celular (*Inside-out integrin signalling*), eventos intracelulares que parecen estar mediados por los dominios citoplasmáticos, influyen en la conformación y afinidad de los dominios extracelulares de las integrinas (Ginsberg et al, 1992). Así, la activación de células T por antígeno o ésteres de forbol conduce a la activación de algunas integrinas $\beta 1$ sin modificar niveles en su superficie (Shimizu et

al, 1990). Señales activadoras transmitidas a través de CD3 o CD2 en la superficie de células T inducen o prolongan la interacción de LFA-1 con su ligando ICAM-1 (Dustin y Springer, 1989b; van Kooyk et al., 1989). CD31 en ciertas subpoblaciones de linfocitos T es un amplificador de la función de LFA-1 y sobre todo de VLA-4 (Tanaka et al., 1992). Desde el exterior celular, la acción de AcM, cationes divalentes, ligandos fisiológicos o factores lipídicos aumentan la adhesión celular generalmente inducendo un cambio conformacional en el receptor (Arroyo et al.; 1992; Hynes 1992; Pardi et al., 1992; Smyth et al., 1993).

Los estudios sobre la regulación de la activación y modulación de afinidad de las integrinas β1, β2 y β3 se han visto favorecidos por el uso de AcM específicos, capaces de detectar epítopos en las subunidades α o β cuya expresión se correlaciona con actividad funcional (Picker et al., 1993; Luque et al., 1996; Dransfield y Hogg, 1989; Diamond y Springer, 1993; Freelinger et al., 1991). Recientemente, se han descrito los AcMs 15/7 (Picker et al., 1993) y HUTS 21 (Luque et al., 1996), que reconocen diferentes epítopos conformacionales activados de integrinas VLA, inducibles en células β1+ por diferentes estímulos como PMA, el AcM anti-β1 activador TS2/16 (Arroyo et al., 1992) y Mn^{++}. (Ver Material y Métodos de Capítulo V y Anexo de Resultados). Estos AcMs constituyen una herramienta útil para constatar la conformación funcionalmente activa de las integrinas β1 que es indispensable para el funcionamiento de la mayoría de ellas.

3.3.4. Transmisión de señales a través de las integrinas β1 y β2

Como hemos visto, las integrinas, a través de sus dominios citoplasmáticos, son capaces de transmitir señales desde el interior celular para variar su porción extracelular con las consiguientes consecuencias funcionales en sus interacciones. Además de esta señalización de *dentro a afuera*, existen múltiples evidencias de que las integrinas en sus interacciones con ligandos celulares o de la MEC transmiten información al interior de la célula. En los últimos años excelentes revisiones compilan esta señalización de *fuera a dentro* (*Outside-in integrin signalling*) que afecta a la diferenciación, proliferación y activación celular así como al patrón de expresión

génica por la célula (Nathan y Sporn, 1991; Makgoba et al., 1992; Hynes 1992, Juliano y Haskill, 1993).

Existen dos mecanismos por los que las integrinas median estas señales y que parecen ser complementarios más que excluyentes. El primero sugiere que las integrinas transmiten señales para la organización del citoesqueleto, regulando la forma celular y la arquitectura interna de la célula. Esto condiciona las capacidades biosintéticas de la célula y así contribuye al crecimiento y diferenciación celular. Pero además, las integrinas pueden actuar como verdaderos receptores capaces de desencadenar señales bioquímicas dentro de la célula. La ocupación del receptor-integrina por su ligando en gran variedad de tipos celulares conduce a la fosforilación en tirosina y a la alcalinización del citoplasma celular. La adhesión de fibroblastos, CE y linfocitos a FN conlleva un aumento del pH citoplásmico que se correlaciona con la estimulación paralela del despliegue (*spreading*) y crecimento celular (Schwartz et al., 1991). Otras señales relacionadas con intregrinas incluyen los niveles de Ca^{++} y de AMPc y la actividad de la bomba Na^{+}/H^{+}. Se piensa que en estos eventos citoplásmicos, las integrinas actúan como receptores sinérgicos con otros receptores para agonistas solubles de estas señales.

En linfocitos T existen múltiples evidencias de integrinas como receptores coestimuladores. Las interacciones de FN con VLA-4 y VLA-5, de LM con VLA-6 y de ICAM-1 con LFA-1 desencadenan señales coestimuladoras con el RCT/CD3 que inducen proliferación de células T tanto vírgenes como de memoria (Davis y Lipsky, 1993). De forma similar, señales transmitidas por VCAM-1 a través de VLA-4 promueven proliferación de células T dependiente de CD3 (Burkly et al., 1991). La unión de FN a VLA-5 induce el factor de transcripción AP-1 necesario para la transcripción de IL-2 (Yamada et al, 1991b).

Los cambios en la inducción de genes mediados por integrinas han sido bien estudiados en monocitos y macrófagos. La estimulación de integrinas con anticuerpos o la adhesión a diferentes glicoproteínas de MEC determinan patrones selectivos de expresión génica, tanto de genes de citoquinas como de metaloproteasas (Juliano y Haskill, 1993; Nathan y Sporn, 1991).

4. ADHESION LINFOCITARIA AL ENDOTELIO VASCULAR

El fenómeno más determinante en el tráfico o migración de linfocitos T es su adhesión al al endotelio vascular. Existen cuatro principios básicos que regulan este tráfico linfocitario (Shimizu et al., 1992): 1) Cada linaje de células inmunes tiene diferentes reglas que regulan su migración; 2) Las células T vírgenes migran fundamentalmente a ganglios linfáticos, mientras que las de memoria migran a tejidos no linfoides; 3) Las células T de memoria tienden a volver preferencialmente al tejido o tejidos relacionados con aquél en el que fueron previamente estimuladas; y 4) la inflamación aumenta el influjo de células T y reduce la selectividad que impera habitualmente en la migración homeostática. En este cuarto principio, el cambio fenotípico inducido en la CE por la inflamación es determinante, y está dirigido a aumentar la eficiencia de la adhesión de células T al endotelio. Por lo tanto, la expresión de muchos de los ligandos que contribuyen a la recirculación normal de linfocitos pueden ser inducidos por la inflamación en lugares donde normalmente no se expresan.

Las CE sometidas a diferentes citoquinas o mediadores inflamatorios dependiendo del tejido y del vaso afectado expresan de forma diferencial y con diferente cinética los receptores de adhesión endotelial (Carlos y Harlan, 1994). Por ejemplo, la tríada IL-1, TNFα y LPS, estimula la expresión de ICAM-1, VCAM-1 y selectina E, pero la expresión *in vitro* de ésta última es mucho menos prolongada, en parte por una rápida internalización de la molécula. Por el contrario la expresión de selectina P se produce por una rápida movilización de los gránulos intracitoplasmáticos, no requiere síntesis de proteínas y es escasamente inducida por citoquinas. Algunos agonistas son más selectivos para algunas proteínas, como IL-4 en la inducción de VCAM-1 o IFNγ en la expresión de ICAM-1. La densidad local de uno u otro receptor en la superficie de las CE y los ligandos expresados por los linfocitos pueden determinar, por tanto, el tipo de interacción de los linfocitos con el endotelio.

Los estudios *in vitro* previos habían demostrado que la adhesión de linfocitos T a CE en cultivo bajo condiciones estáticas estaba mediada por diferentes vías de adhesión, dependiendo del

estado de activación de ambos tipos celulares (Shimizu 1991b). En la adhesión de células CD4+ no activadas al endotelio inflamado se habían implicado al menos 3 pares de ligando/receptor: LFA-1/ICAM-1, VLA-4/VCAM-1 y SLe^X o carbohidratos relacionados/ELAM-1. Estudios posteriores *in vitro* e *in vivo*, bajo condiciones de flujo, han revelado que múltiples pares ligando-receptor pueden actuar secuencialmente o solapándose para llevar a cabo las diferentes fases de lo que se ha llamado "la cascada de adhesión" (Butcher 1991, Shimizu et al, 1992, Springer 1994). Este modelo "multifases" propone que los miembros de la familia de las selectinas median las interacciones iniciales a través de sus ligandos carbohidratados, seguidas de una adhesión firme y subsecuente diapedesis, que requieren la activación de las integrinas para unirse a sus ligandos endoteliales en la superfamilia de las Ig. Sin embargo, estudios más recientes en condiciones de flujo, han introducido cambios sobre la función de cada par ligando-receptor en las interacciones de linfocitos T con el endotelio estimulado. La figura 5 muestra un esquema sobre los diferentes pasos de adhesión de linfocitos al endotelio con las moléculas implicadas.

4.1. Fase 1: Contacto inicial y adhesión reversible o rodamiento

Los linfocitos deben ser reclutados a los lugares de inflamación en condiciones de flujo. Bajo las condiciones de estrés lateral, la rápida cinética adhesiva de las selectinas, su estructura flexible y su localización distal en las microvellosidades de las células hacen posible el primer contacto con el endotelio seguido por el rodamiento por la pared del vaso, en una sucesión de adhesiones y desuniones consecutivas sobre las CE. Hasta hace poco tiempo, se pensaba que sólo las selectinas eran capaces de sustentar este primer paso en condiciones no estáticas, pero recientes investigaciones demuestran que las interacciones $\alpha4\beta1$/VCAM-1, $\alpha4\beta7$/VCAM-1 y $\alpha4\beta7$/MAd-CAM-1 también están involucradas en el primer contacto y rodamiento de células linfoides sobre el endotelio (Berlin et al, 1995). Estos autores muestran además, mediante microscopía electrónica de barrido, la localización de $\alpha4\beta7$ en las microvellosidades de las células linfoides en los lugares de contacto inicial. Estudios previos *in vitro* bajo condiciones de flujo, demuestran la capacidad de linfocitos T para detenerse y rodar sobre monocapas de células transfectadas bien con ELAM-1 o con VCAM-1 (Jones et al, 1994). La selectina P es capaz de mediar la unión inicial de linfocitos

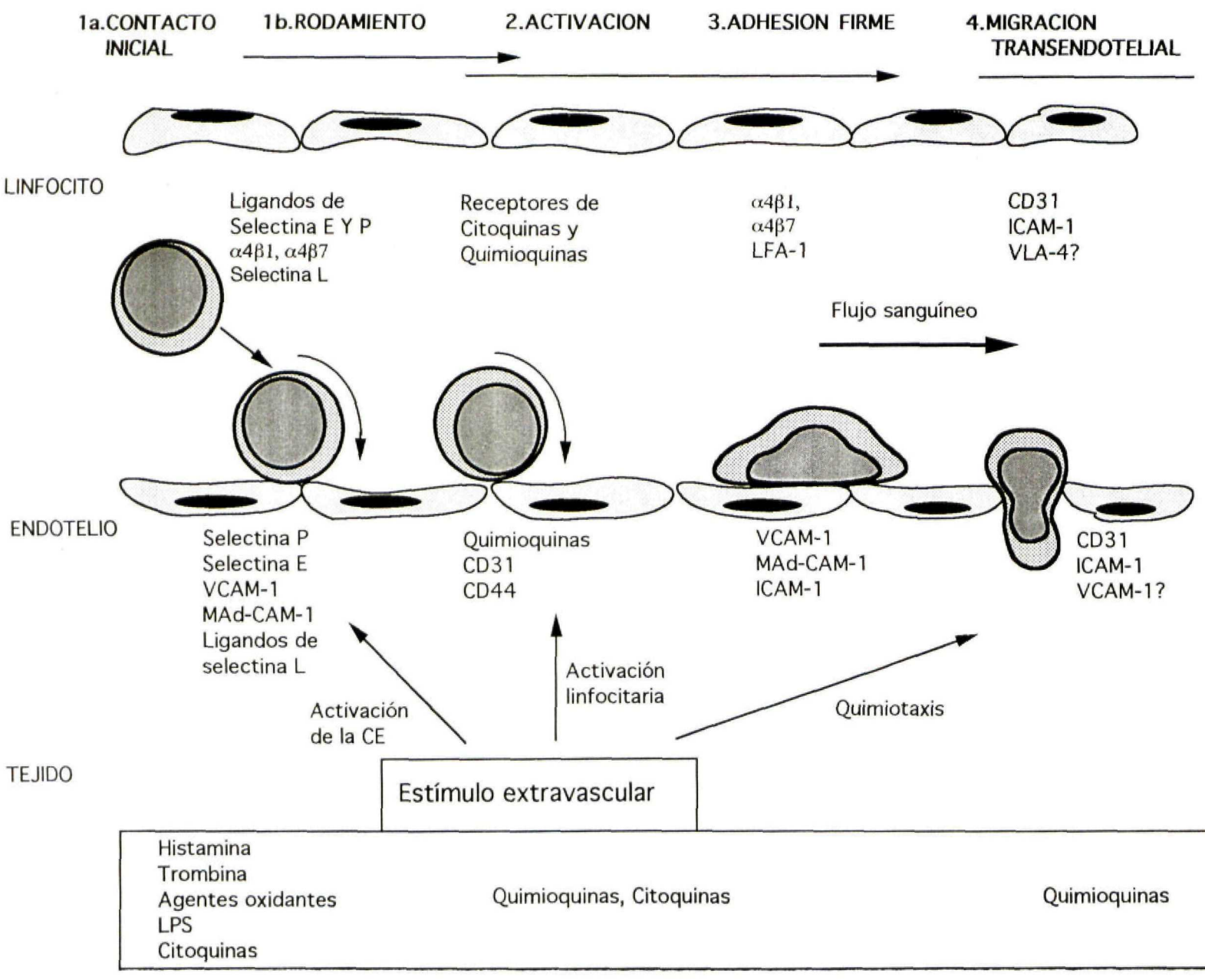

Fig 5. Las interacciones adhesivas de linfocitos con el endotelio se desarrollan en fases consecutivas y a veces solapadas: adhesión inicial transitoria, activación y adhesión firme, y migración transendotelial. En la figura se recogen los distintos componentes de linfocitos, *células endoteliales* y tisulares que participan en cada fase.

$CD4^+$ a HUVEC estimuladas con TNFα en condiciones de flujo, pero el rodamiento se sustenta tanto por la selectina P como por VCAM-1 a través de su ligando VLA-4 (Luscinskas et al, 1995). Sin embargo, VCAM-1 no parece involucrado en la adhesión inicial de linfocitos a HUVEC estimuladas con IL-1 ni con IL-1+IL-4 (Jones et al, 1994). La participación de la selectina L en el *rolling* de linfocitos sólo se ha descrito en HEV de ganglios linfáticos intestinales a través de MAd-CAM-1 (Berg et al., 1993). Estructuras similares a HEV en tejidos inflamados se tiñen con el AcM MECA-79, que reconoce ligandos en HEV para la selectina-L, por lo que también esta selectina parece estar involucrada en el reclutamiento de linfocitos a lugares de inflamación (Girard y Springer,1995).

4.2. Fase 2. Activación linfocitaria

Las interacciones iniciales, transitorias, que median el rodamiento de los linfocitos son reversibles a menos que la célula sea activada, en cuyo caso se detiene para adherirse de forma definitiva al endotelio. Sin embargo estas interacciones son suficientes para frenar la velocidad de la célula, prolongando el contacto con la pared vascular y favoreciendo su exposición a factores quimiotácticos y activadores producidos localmente. Estos factores activadores pueden derivar de las propias células que componen el tejido, de leucocitos infiltrantes, de microorganismos y del propio endotelio. Recientemente se ha descrito una familia de citoquinas quimiotácticas, denominadas **quimioquinas**, con especifidad para distintos grupos de leucocitos, que son los candidatos idóneos para la activación de las integrinas dentro de la microvasculatura (Rot, 1993; Schall y Bacon, 1994). Se han definido varias subfamilias según su estructura, que se correlacionan con su capacidad para atraer diferentes tipos de leucocitos: las quimioquinas C-C parecen afectar principalmente a neutrófilos (y en alguna medida a linfocitos); las C-X-C actúan sobre monocitos, linfocitos, eosinófilos y basófilos y la quimioquina C (Linfotactina) sólo sobre linfocitos. Además, algunas de ellas como MIP1α y -1β, RANTES e IP10 son capaces de incrementar las propiedades adhesivas de los subgrupos de linfocitos para las que son quimiotácticas (Tanaka et al, 1993a; Taub et al., 1993a; Taub et al., 1993b). Es interesante reseñar que RANTES, MIP-1α y MCP-1 son capaces de atraer selectivamente linfocitos T de memoria (Schall et al., 1990; Carr et al., 1994).

La familia de las quimioquinas se define por su estructura, pero comparten 3 propiedades funcionales comunes que las convierten en candidatos idóneos para activar la adhesión fisiológica : 1) Atraen selectivamente uno o más grupos de células mieloides; 2) la producción y secreción de la mayoría de las quimioquinas por las células productoras (linfocitos activados, monocitos, fibroblastos, CE) se induce por estímulos proinflamatorios como LPS, TNF α o IL-1, presentes en abundancia en los focos inflamatorios; 3) todas las quimioquinas que se han testado inducen infiltrados inflamatorios cuando se injectan intradérmicamente en animales (Oppenheim et al., 1991; Murphy, 1994). Por lo tanto, estos agentes quimiotácticos producidos por células del tejido o por las propias CE, unidos a proteoglicanos de MEC u otras moléculas presentadoras en la superficie endotelial (Tanaka et al., 1993b) podrían formar gradientes quimiotácticos estables en fase sólida, capaces de atraer selectivamente y activar a las células durante el rodamiento.

Otra posible vía de activación podría estar mediada por interacciones de los linfocitos con otros receptores como CD44 y CD31 que son capaces de modular la función de las integrinas β1 y b2(Tanaka et al, 1992; Koopman et al, 1990).

4.3. Fase 3. Adhesión firme

La acción de las quimioquinas está mediada por receptores en la superficie de leucocitos, acoplados a proteínas G, que son moléculas intermediarias cruciales en la transducción de señales desde el exterior (Murphy 1994). La activación aumenta de forma dramática la adhesividad de linfocitos a la CE mediante un aumento de afinidad de las integrinas β1 y β2 mediado por cambios conformacionales en las moléculas (Lucinskas y Lawer, 1994; Rot, 1993) . Por lo tanto, aunque moléculas preactivadas de VLA-4 sean capaces de mediar las interacciones iniciales de baja afinidad con el endotelio, su activación determina la unión firme a las CE en conjunción con LFA-1.

4.4. Fase 4. Migración transendotelial

El paso de los linfocitos a través de las uniones entre las CE requiere primero una disminución de la adhesión, mediada por el carácter transitorio del aumento de función de las

integrinas, que permita de nuevo a la célula el movimiento hacia la membrana basal y el intersticio circundante. Este proceso de migración transendotelial parece mediado fundamentalmente por las interacciones LFA-1/ICAM-1 y, en aquellos subtipos linfocitarios en los que se expresa, las interacciones homotípicas de CD31 con el endotelio (Carlos y Harlan, 1994).

El gradiente quimiotáctico establecido por las quimioquinas desde el tejido productor hasta el endotelio seguiría guiando la diapedesis y migración de la célula hasta el espacio subendotelial.

5. INTERACCIONES DE LINFOCITOS T CON SINOVIOCITOS TIPO FIBROBLASTO

La acumulación de linfocitos T en los infiltrados inflamatorios y la hiperplasia de la capa de sinoviocitos son dos características constantes en la histopatología de la AR. Los linfocitos T circulantes alcanzan la MS a través de su interacción con el endotelio, y es concebible que, una vez en el tejido, desarrollen interacciones con la MEC y con otros tipos celulares. Además de las interacciones con CE y macrófagos, estudios de microscopía electrónica en la AR han detectado contactos de células T con fibroblastos sinoviales (Kobayashi y Ziff, 1973). Estudios *in vitro* evidencian interacciones estimuladoras recíprocas entre células T y fibroblastos sinoviales. Una suspensión de linfocitos y células sinoviales procedentes de la MS de pacientes con AR, cultivados en presencia de antígenos de micobacterias e IL-2, son capaces de producir un tejido inflamatorio organizado con MEC y características histológicas de *pannus* (Holoshitz et al., 1991) A pesar de estas evidencias, hasta ahora sólo se han estudiado interacciones de STF (Sinoviocitos tipo fibroblasto) con células T de SP, por lo que las MA que median estas interacciones *in vivo* en la MS no son del todo conocidas.

Los primeros estudios realizados con AcM involucraban a la molécula LFA-3 de STF en cultivo, en la adhesión de células T de SP a través de su ligando CD2 (Haynes et al., 1988). Investigaciones posteriores no consiguieron demostrar la participación de estas moléculas pero defendían la vía mediada por ICAM-1 (Krzesicki et al, 1991), una molécula con alta expresión en las células del *lining* sinovial de AR (Hale et al., 1989; Koch et al ., 1991). La expresión basal de esta molécula aumenta cuando se estimulan STF en cultivo con las citoquinas IFNγ, TNFα, IL-1β o IL-4 (Krzesicki et al., 1991; Morales Ducret et al., 1992). La presencia de su contrarreceptor en linfocitos, LFA-1(CD11a/CD18), ha sido bien documentada en linfocitos del infiltrado de AR (Hale et al., 1989; Cush et al 1988). Sin embargo, esta adhesión sólo podía ser inhibida parcialmente por AcM anti-CD11a y anti-CD18, lo que sugería la existencia de otras vías de adhesión.

Como hemos visto en apartados anteriores (Ver epígrafe 3.2. de Introducción), otra molécula de la superfamilia de las inmunoglobulinas, VCAM-1, se expresa constitutivamente en STF y su expresión puede ser también regulada *in vitro* por las citoquinas IL-1β, IL-4, TNFα e IFNγ (Morales Ducret et al, 1992). En este sentido, se ha descrito que el aumento de expresión de VCAM-1 inducido por IL-4 en STF se correlaciona con una adhesión aumentada de células T de SP (Shimada et al., 1994). VCAM-1 interacciona con VLA-4, una integrina cuya expresión y función está aumentada en células T de memoria (Shimizu et al., 1990). Por lo tanto, la presencia de grandes cantidades de citoquinas proinflamatorias en la MS reumatoide podía estar regulando estas interacciones a través de una expresión alta y mantenida de los ligandos en STF, pero su efecto sobre las células T no ha sido estudiado hasta el momento. Como expondremos en el capítulo III de Resultados, las células T de LS Y MS de AR se adhieren más eficazmente a VCAM-1 purificado que sus correspondientes de SP. La importancia de estas interacciones se refuerza por la capacidad de VCAM-1 de desencadenar señales coestimuladoras en linfocitos T a través de VLA-4(Burkly et al., 1991). Por lo tanto la interacción VLA-4/VCAM-1 en la unión de células T de compartimentos sinoviales a STF podría ser especialmente relevante *in vivo*.

OBJETIVOS

La acumulación y persistencia de linfocitos T en la MS de AR es uno de los factores determinantes en la patogenia de la enfermedad. En este microambiente sinovial, bajo múltiples factores reguladores, se suceden múltiples interacciones de linfocitos T con otras células y con la MEC, que conducen a la destrucción articular. Las integrinas constituyen una importante familia de receptores de adhesión celular tanto para proteínas de MEC como para ligandos celulares, con capacidad para regular su expresión y función de forma rápida y dinámica. Bajo estas premisas nos planteamos los siguientes objetivos:

1) Conocer la expresión de diferentes integrinas VLA (α1-5/β1) en las células T de diferentes compartimentos de pacientes con AR.

2) Estudiar el comportamiento *in vivo* de los receptores de fibronectina VLA-5 y VLA-4 en cuanto a su expresión y función en linfocitos T de AR, y los posibles mecanismos de regulación.

3) Estudiar la interacción de linfocitos T de Artritis Reumatoide con los ligandos endoteliales ELAM-1 y VCAM-1, su regulación y posible inducción de señales en los linfocitos T.

4) Investigar las MA involucradas en las interacciones de linfocitos T de AR con sinoviocitos tipo fibroblasto, y la regulación por citoquinas de estas interacciones en ambos tipos celulares.

5) Estudiar la presencia *in vivo* de conformaciones activas de las integrinas β1 en AR y otras patologías inflamatorias crónicas, y los mecanismos fisiológicos que pueden regular su aparición.

MATERIAL Y METODOS

Todos los materiales y métodos utilizados en esta tesis se describen en los apartados correspondientes de cada uno de los capítulos de Resultados.

RESULTADOS

CAPITULO I

EXPRESION DE LOS RECEPTORES DE LA FAMILIA VLA EN ARTRITIS REUMATOIDE. EVIDENCIA DE LA REGULACION *IN VIVO* DEL RECEPTOR DE FIBRONECTINA VLA-5 EN LINFOCITOS T DEL LIQUIDO SINOVIAL

La adhesión de células T a proteínas de MEC a través de receptores de la familia de integrinas VLA, es un fenómeno crucial en el tráfico de linfocitos, su localización en un tejido e incluso contribuye a su función proinflamatoria. Se ha investigado por citometría de flujo la expresión de distintos heterodímeros VLA (α1-5 y la subunidad β1 común) en linfocitos T de SP y LS obtenidos de pacientes con AR . Las células T del LS muestran muy baja expresión de las subunidades α2 y α3, moderada de α1, y alta expresión de α4 y β1, siendo el incremento de las 3 últimas significativo respecto a SP (Tabla 1). Por el contrario, α5 se expresa en ambos compartimentos sin diferencias significativas en porcentaje de células positivas ni en intensidad de fluorescencia (Tabla 1 y Fig. 1). Las células del LS están activadas como demuestra su expresión del antígeno de activación temprana AIM (CD69), prácticamente ausente en linfocitos T de SP (Tabla 1 y Fig 1) Mediante técnicas de inmunohistoquímica se ha estudiado la distribución tisular de estas moléculas en la MS (Fig. 2). Las células del *lining* sinovial hipertrófico se tiñen con AcM frente a las subunidades β1 y todas las α, con excepción de α4. Por el contrario, el AcM HP2/1 (α4) tiñe intensamente la mayoría de los linfocitos T del infiltrado (tinción con anti-CD3 no mostrada). La tinción con anti-β1 muestra expresión difusa pero menos intensa en los infiltrados linfoides. Un número más reducido de linfocitos infiltrantes muestran expresión de α1 y α5. El endotelio presenta una tinción llamativa con anti-β1 y además expresa α1,2,3 y 5.

Los estudios funcionales se centran en el comportamiento de VLA-5 como receptor de FN para las células T de AR. Un porcentage significativamente superior de células T del LS se adhieren al fragmento de 80 KD de la FN humana (que contiene la secuencia RGD), respecto a sus correspondientes de SP (Fig. 3). Esta adhesión puede ser bloqueada por un AcM anti-β1, y está específicamente mediada por VLA-5, como se demuestra por la inhibición con el AcM anti-α5 y con péptidos RGD (Fig. 3). Esta capacidad de adhesión aumentada de las células T de LS parece ser independiente del nivel de expresión del receptor VLA-5 y se correlaciona con el estado de

activación de las células T sinoviales como demuestra su expresión del antígeno de activación linfoide CD69 (AIM). Por lo tanto, demostramos que la función de los receptores de FN VLA-5 en linfocitos T de AR está regulada *in vivo*. La presencia de múltiples receptores VLA en la MS de AR sugiere la implicación de las proteínas de MEC en la perpetuación del proceso inflamatorio.

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VLA family in rheumatoid arthritis: evidence for *in vivo* regulated adhesion of synovial fluid T cells to fibronectin through VLA-5 integrin

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SUMMARY

Adhesion of T cells to extracellular matrix (ECM) proteins through VLA integrin receptors is crucial for lymphocyte trafficking, tissue localization and inflammatory function. We have investigated the expression of different VLA integrins (VLA-1–5) on peripheral blood (PB) and synovial fluid (SF) T lymphocytes from patients with rheumatoid arthritis (RA). Their expression on different cell types from synovial membrane (SM) is also reported. The role of VLA-4 fibronectin (FN) receptors in the interaction of activated SF T cells from RA patients with a 38-kD fragment of FN has been previously demonstrated. Here we have focused functional studies on VLA-5 as an alternative FN receptor for RA T cells. A significant higher proportion of SF T cells were able to bind to an 80-kD fragment of FN, containing the Arg–Gly–Asp (RGD) cell binding site, compared with PB T cells. This attachment was almost completely inhibited by anti-VLA-5 MoAbs as well as by RGD peptides. This enhanced capability by SF T cells appears to be independent of the level of the surface expression of the receptor and correlates better with their activation state as determined by the expression of the activation molecule AIM (CD69). The evidence for the expression of VLA heterodimers on both SF and SM cells from RA patients suggests the possible implication of ECM proteins in mediating and perpetuating inflammation *in vivo*.

Keywords rheumatoid arthritis VLA T lymphocytes fibronectin receptors

INTRODUCTION

The integrin family contains numerous cell surface receptors that have been shown to mediate cell–cell interactions as well as binding of cells to extracellular matrix (ECM) proteins [1–3]. A considerable proportion of research has been focused over the past few years on the $\beta 1$ integrin subfamily, also termed VLA (very late activation antigen) family, derived from the diverse capability of its members to function as receptors for ECM proteins [4]. These cell–ECM interactions can influence the trafficking and probably the anchoring of leucocytes into specific tissues or target organs. Several $\beta 1$ integrins recognize fibronectin (FN). VLA-5 is well recognized as the prototype FN receptor [5]. The VLA-4 integrin has been also described as a receptor for FN [6,7], beside its involvement in different cell–cell adhesion functions [8–12]. The binding of VLA-3 to FN is under controversy [13,14]. Recently, integrins $\alpha V\beta 1$ on neuroblastoma cells and $\alpha V\beta 3$ on melanoma cells have also been described to bind FN [15–17]. The interaction of these molecules with FN is displayed through different domains of this ECM protein. Thus, VLA-3, VLA-5, and $\alpha V\beta 3$ bind to a segment containing Arg–Gly–Asp (RGD), called central cell attachment domain, which is contained in an 80-kD tryptic fragment of plasma FN, whereas VLA-4 interacts with the connecting segment 1 (CS-1) included in a 38-kD proteolytic fragment [6].

Among hematopoietic cells, VLA-5 is constitutively expressed by a subset of T lymphocytes, platelets, monocytes, and different B and T cell lines [4,6]. Adhesion of resting human T cells to intact FN is poor unless they are activated [18]. In this sense, the increase in VLA-5-mediated T cell binding to FN, without any associated change in VLA-5 expression, is observed after activation of $CD4^+$ T cells [18].

Blocking experiments using MoAbs suggest that the interaction of T cells with FN through VLA-4 and VLA-5, provides a costimulatory signal in CD3-mediated T cell activation [19–21], and thus could implicate the ECM protein FN in the regulation of T cell responses.

So far, few studies of human pathology evaluating the expression of VLA proteins at inflammatory sites have been reported. Previously, in accordance with data from other

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investigators, we described the presence of VLA-1 on activated T cells from rheumatoid synovial fluid (SF) [22–24]. Furthermore, we have previously demonstrated the VLA-4-mediated interaction of activated SF T cells from patients with rheumatoid arthritis (RA) with a 38-kD fragment of FN [25]. Here we have analysed, as a first step, the expression of different VLA members (VLA-1–5) on peripheral blood (PB) and SF T cells from RA patients, as well as their distribution into inflamed synovial membrane (SM). In addition, we have investigated in detail the functional capability of VLA-5 as a FN receptor for T cells *in vivo*. We have demonstrated an augmented VLA-5-mediated adhesiveness to FN by SF T cells, suggesting the existence of activation-mediated regulatory mechanisms of the VLA-5–FN interactions at inflammatory sites in this disease.

PATIENTS AND METHODS

Patients

Three men and 10 women with RA (median age 48·5 years, range 21–76) were investigated. All of them fulfilled criteria for the diagnosis of RA according to the American College of Rheumatology [26]. The median duration of disease was 7·3 years (range 8 months to 14 years). All patients were taking non-steroidal anti-inflammatory drugs. In addition, four of them were taking gold derivatives, one anti-malarial drugs, one D-penicillamine, one oral methotrexate and only one patient was under low doses of corticosteroid therapy.

Preparation of purified T lymphocytes

PB and SF were collected at the same time into heparinized tubes, and mononuclear cells (MNC) were isolated by Ficoll–Hypaque gradient centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden). For purification of T cells, adherent cells were removed from MNC by culturing on plastic Petri dishes for 45 min at 37 C. Briefly, non-adherent cells were placed on a 600 mg nylon-wool column pre-incubated for 30 min with RPMI 1640 (Flow, Irvine, UK) supplemented with 10% FCS (GIBCO, Grand Island, NY), 2 mM glutamine and 50 μg/ml penicillin/streptomycin. MNC were then incubated in the column for 45 min at 37 C. Cells were eluted with 20 ml of RPMI 1640. The purified T lymphocyte fractions contained ⩾90% of CD3, ⩽6% monocytes and ⩽1% B cells, as determined by expression of the CD3, Mo-2 (CD14) and B1 (CD20) antigens, respectively.

Monoclonal antibodies

The activation marker AIM/CD69 (activation inducer molecule) was studied with the TP1/55 MoAb [27]. MoAbs used to study VLA heterodimers were directed against the β1 common chain (TS2/16 and Lia1/2) [28], and towards the different α subunits: TS2/7 (anti-α1) [28], P1E6 (anti-α2) [29], P1B5 (anti-α3) [29], HP2/1 (anti-α4) [30] and P1D6 (anti-α5) [29].

In order to assess the purity of T cell preparations, cells were stained with SPV-T3b (anti-CD3) [31], Bear-1 (anti-CD11b) [32], and BC-1 (anti-CD20) [33]. Anti Mo-2 (CD14) was kindly provided by J. E. de Vries (Unycet Lab, Dardilly, France). The MoAb D3/9 specific for the leucocyte common antigen (T200/CD45) [34], was used as control. X63 MoAb (IgG1), used as negative control, is the immunoglobulin secreted by the mouse myeloma cell line P3–X63.

Flow cytometry analysis of T cell surface antigens

Viable cells ($1–5 \times 10^5$) were suspended in 50 μl aliquots of PBS, pH 7·4. Specific mouse MoAb (1 μg in 50 μl) was added and cells were incubated for 30 min at 4 C, then washed twice and incubated with saturating amounts of fluorescein-conjugated $F(ab')_2$ goat anti-mouse immunoglobulin (Dakopatts, Copenhagen, Denmark). After three washes, fluorescence was measured using an EPICS C flow Cytometer (Coulter, Hialeah, FL). Fluorescence intensity for different MoAbs was determined on a logarithmic scale, and data for mean fluorescence intensity (MFI) were converted to arbitrary linear units. Background fluorescence for irrelevant MoAb P3X63 was determined for each cell population and subtracted. The specific percentages of positive cells for different MoAbs were obtained by subtracting the number of background cells that were non-specifically stained with the MoAb P3X63.

Immunoperoxidase staining of tissue sections

Synovial samples were obtained by surgical synovectomy from three RA patients, frozen in OCT (Ames Miles, Elkhart, IN) and stored at −80 C. Tissue sections were stained by an indirect immunoperoxidase method as described [35]. Briefly, 4 μm acetone-fixed sections were sequentially incubated with MoAb culture supernatants and peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts); each incubation was followed by three washes. The reaction was developed with Graham–Karnovsky medium containing 0·5 mg/ml of DAB and hydrogen peroxide. Sections were counterstained with Carazzi's haematoxylin followed by dehydration and mounting by routine methods.

Cell attachment analysis

Human FN 80-kD proteolytic fragment (from Dr A. García-Pardo, CIB, CSIC, Madrid) and type I COL (Sigma, St Louis, MO) were applied to 96-well flat-bottomed microtitre plates (Linbro, Flow) (40 μg/ml, 0·1 ml/well) in CO_3HNa 0·1 M at 4 C overnight. Non-specific binding sites were saturated with RPMI 1640/1% human seroalbumin for 2 h at 37 C. Purified T cells isolated from PB and SF were added (125 000 cells/well) in 0·1 ml of RPMI 1640 and incubated at 37 C and 5% CO_2. After 30 min, plates were washed with RPMI 1640 several times and examined in an inverted microscope by at least two different observers. Each condition was performed in duplicate. In inhibition conditions, cells were incubated for 30 min at 4 C with: (i) 25% final volume of anti-α5 P1D6, anti-α4 HP2/1 and anti-β1 Lia1/2 hybridoma culture supernatants; (ii) RGDS synthetic peptides (Sigma) (500 μg/ml) and added to the wells. As control conditions, cells were incubated with RPMI 1640 1% human seroalbumin, D3/9 (anti-CD45) hybridoma culture supernatant and RGES synthetic peptides (Sigma) (500 μg/ml). Within each well cells from at least three different fields were counted and referred to a non-washed well (100% or maximum binding).

Statistical analysis

Values of PB and SF T cell samples from RA patients were analysed using Student's *t*-test for paired samples.

Table 1. Expression of adhesion and activation antigens on purified T cells from peripheral blood (PB) and synovial fluid (SF) of rheumatoid arthritis (RA) patients

	Healthy donors ($n=6$)		RA patients ($n=12$)					
	PB		%			MFI		
MoAb	%	MFI	PB	SF	*P*	PB	SF	*P*
Anti-VLA1α (TS2/17)	4±1	1±0·5	8±3	18±4	*	1±1	3±1	†
Anti-VLA2α (P1E6)	4±1	1±0	6±1	9±3	NS	2±1	3±1	NS
Anti-VLA3α (P1B5)	1±0	0	5±2	11±4	NS	2±1	5±2	NS
Anti-VLA4α (HP2/1)	43±3	14±3	48±6	73±3	‡	15±3	23±3	*
Anti-VLA5α (P1D6)	30±3	4±1	38±9	39±7	NS	7±2	7±1	NS
Anti-VLA β1 (TS2/16)	60±9	23±4	65±5	88±2	‡	29±7	46±7	‡
AIM (TP1/55)	3±2	1±0	6±2	48±6	‡	2±1	14±2	‡

Values are the percentages of T cells positive for each antigen (mean ± s.e.m.). Data were obtained by subtracting the number of background cells that were nonspecifically stained with the control MoAb X63. Data for mean fluorescence intensity (MFI) are expressed in arbitrary linear units (mean ± s.e.m.). Data shown are after subtraction of background fluorescence of cells in the presence of irrelevant MoAb X63. Statistical significance: * $P<0.01$; † $P<0.05$; ‡ $P<0.001$; NS, not significant.

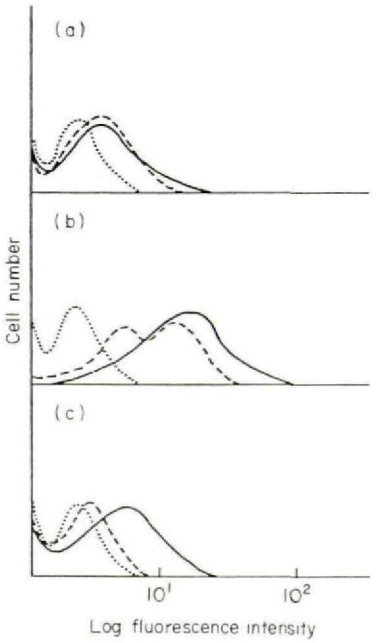


Fig. 1. Immunofluorescence flow cytometry analysis of adhesion (α5 and β1) and activation (AIM/CD69) antigens on synovial fluid (solid line), and peripheral blood (dashed line) purified T lymphocytes from a representative patient with rheumatoid arthritis. Cells were labelled with (a) P1D6 MoAb (anti-VLA α5); (b) TS2/16 MoAb (anti-VLA β1); (c) TP1/55 MoAb (anti-AIM), and the negative control X63 MoAb (dotted line).

RESULTS

Expression of VLA proteins on PB and SF T cells from RA patients

The distribution of distinct α subunits (VLA-1–5) and β1 chain was analysed on T cells from PB and SF compartments by flow cytometry. Results obtained from 12 RA patients are summarized in Table 1.

In agreement with previous reports [22–24], SF T cells expressed low to moderate amounts of α1 subunit, but in a significant higher level than PB T cells, where there is slight or no detectable expression (Table 1). The expression of α2 and α3 on resting PB T cells was actually negligible, and only a slight increase, without significant differences, was found in SF. It is apparent from data in Table 1 that there is a higher expression of α4 subunit on SF T cells compared with PB compartment from the same patients. By contrast, we have not found significant differences in the expression of α5 molecules between PB and SF T lymphocytes, in terms of percentage of positive cells and MFI (see profiles of flow cytometry from a representative patient in Fig. 1).

Common β1 subunit is clearly represented on T cells from PB with a significant increment in its expression on SF T cells (Table 1, Fig. 1).

The levels of surface expression and proportion of positive cells for different VLA-1–5 α and β1 subunits on PB T cells from both RA patients and healthy donors were generally in the same range (Table 1). Expression levels of α2 and α3 subunits were slightly higher on PB T cells from patients but were in all cases very low or undetectable. However, although a subpopulation of PB T lymphocytes obtained from healthy individuals expressed α5, a higher expression of this antigen was observed in RA patients.

Distribution of VLA proteins in synovial membrane from RA patients

Immunoperoxidase staining of frozen tissue sections from SM of three RA patients was performed for the different α (1–5) and β1 reactivities (Fig. 2). All synovial biopsy samples contained several lymphocytic aggregates in the subsynovia, usually surrounding blood vessels. Most of the cells in these aggregates exhibited T phenotype, as determined by CD3 MoAb staining (data not shown).

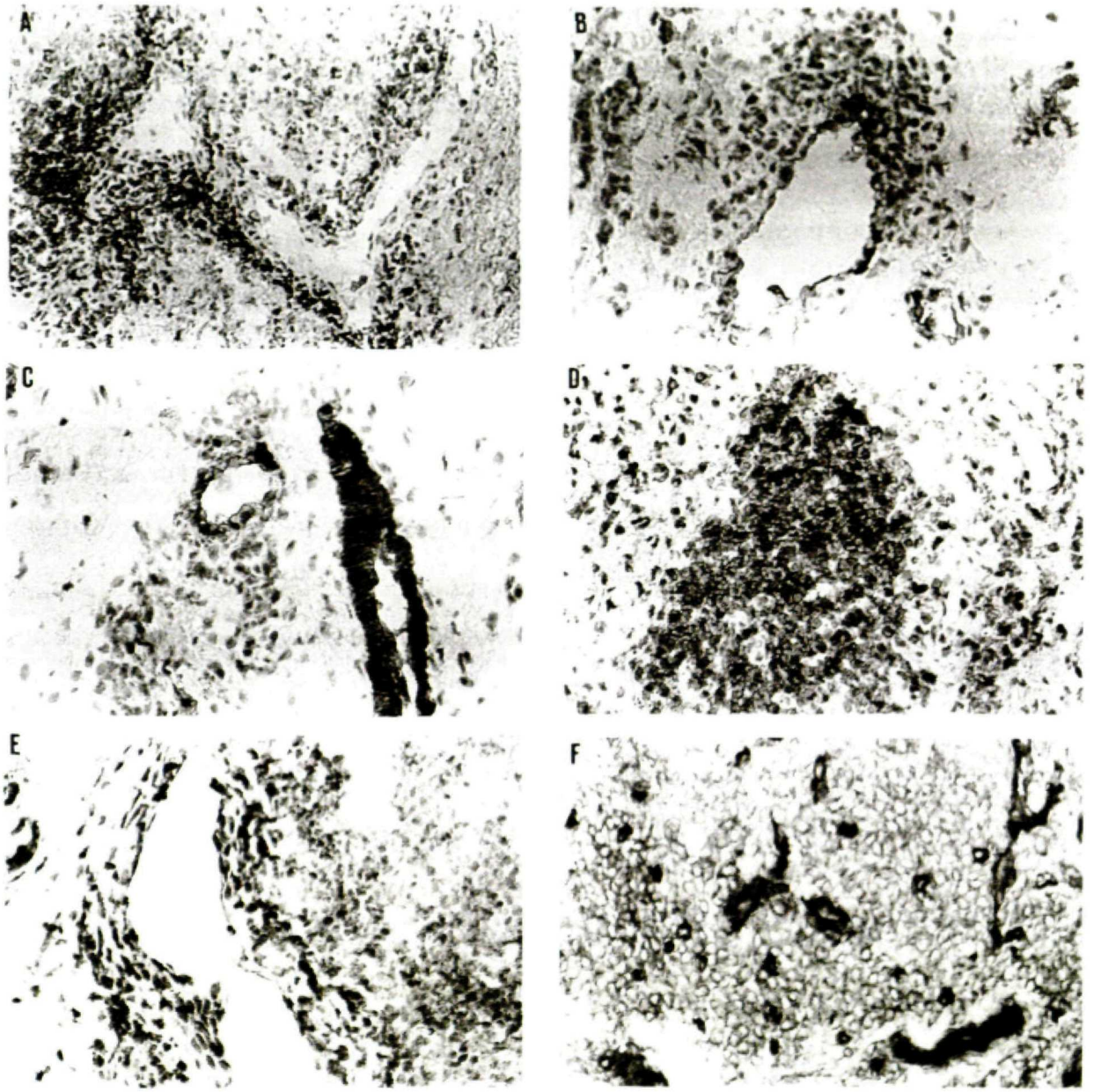


Fig. 2. Immunohistologic identification of the reactivity of MoAbs against VLA α and β molecules within inflamed synovial membrane. Frozen sections of synovial tissue obtained from patients with rheumatoid arthritis were stained with the indicated MoAb by immunoperoxidase technique: (A) TS2/7 (anti-α1); (B) P1E6 (anti-α2); (C) P1B5 (anti-α3); (D) HP2/1 (anti-α4); (E) P1D6 (anti-α5); and (F) TS2/16 (anti-β1). Magnification: (A), $\times$200; B–F, $\times$400.

One of the most striking features on tissue staining was the widespread distribution of different VLA α and β1 chains on synoviocytes within hyperplastic synovial lining, with the exception of α4. Opposed to this lack of reactivity on synovial cells, anti-α4 MoAb stained the majority of T lymphocytes in inflammatory infiltrates from the subsynovia (Fig. 2D). In addition, more reduced numbers of T cells stained with anti-α1 (Fig 2A) and anti-α5 MoAb (Fig. 2E). Both anti-α1, α2, α3 and α5 MoAbs showed positive reaction on endothelial cells from blood vessels (Fig. 2A, B, C, E). The anti-β1 MoAb TS2/16 stained strongly synoviocytes and blood vessels, showing a weak diffuse positive reaction on lymphocytes (Fig. 2F).

Expression and function of VLA-5 FN receptor on SF and PB T cells from RA patients

To investigate whether VLA-5 integrin could play a significant role in RA we studied its expression and function on T lymphocytes from PB and SF of RA patients.

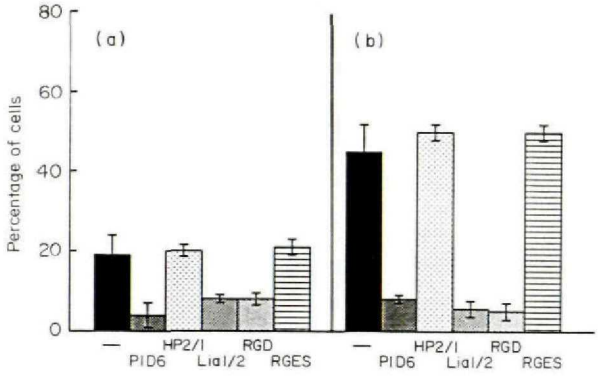


Fig. 3. Comparison of the functional capacity of purified T cells from peripheral blood (a) and synovial fluid (b) compartments, for binding to the 80-kD fibronectin fragment. Adhesion to this FN fragment in the presence of anti-α5 PID6, anti-α4 HP2/1, anti-β1 Lia1/2 MoAbs, RGDS and RGES peptides is also represented. Values are mean ± s.e.m. of data obtained from at least three experiments.

As shown in Table 1, T cell expression levels of α5 subunit in both compartments were quite similar. It is worth emphasizing that weak or no increments in the percentage of positive cells were detected in the SF compartment and no significant differences were observed when paired samples from PB and SF were compared. Those results were in agreement with data obtained from PB and SF T cells of four patients with Reyter's syndrome, psoriatic arthritis, HLA-B27-related oligoarthritis and HLA-B27 reactive arthritis. Little increments in the proportion of α5-positive cells were observed on SF T cells with minimal changes in fluorescence intensity (data not shown).

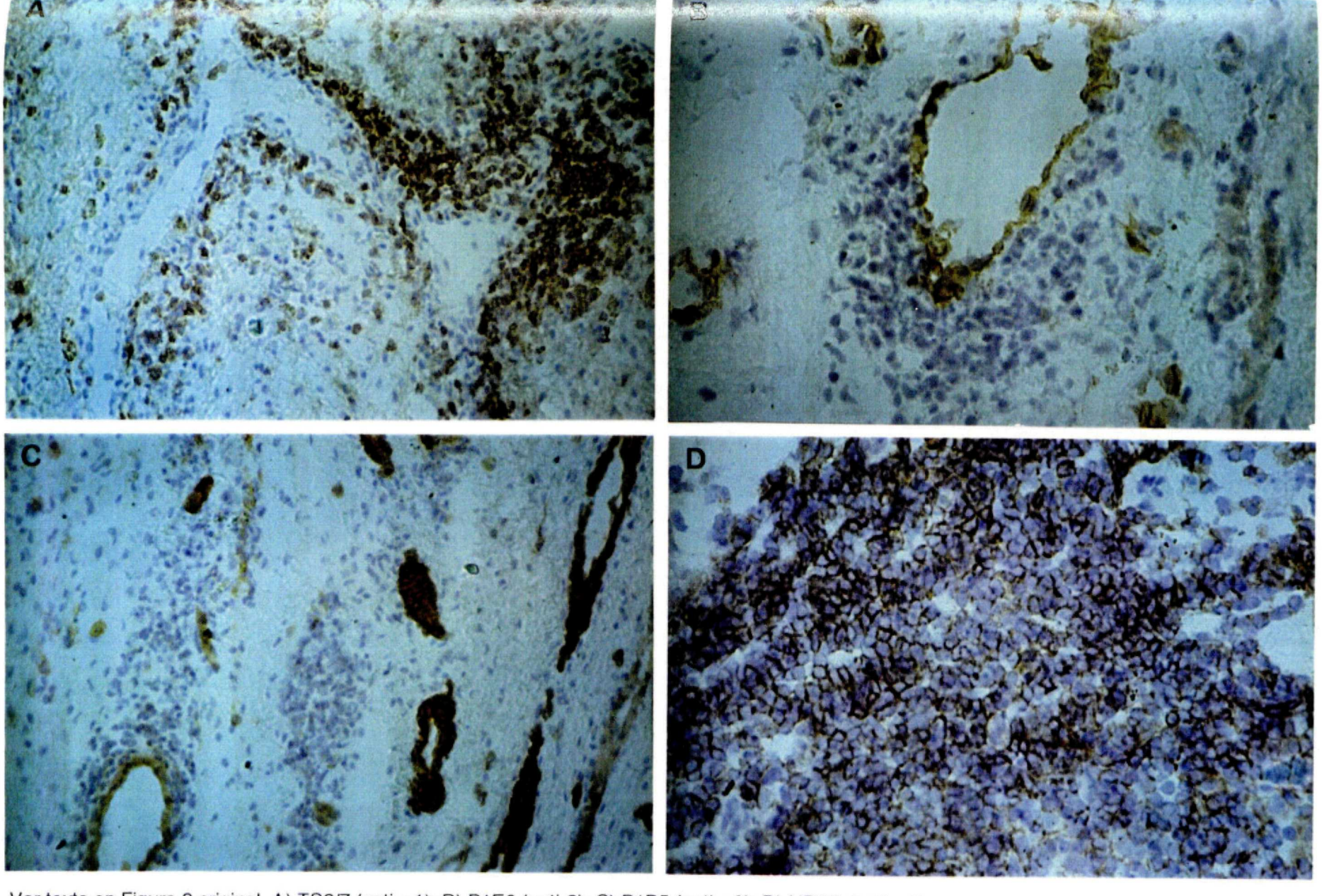
We also studied the activation state of T cells from both compartments by measuring the expression of the activation antigen AIM (CD69). Our results showed the presence of elevated amounts of AIM on SF T cells whereas low or undetectable expression of this molecule was observed on PB T cells (Table 1 and Fig. 1). Similarly, a significant proportion of the $CD3^+$ lymphocytes infiltrating the SM stained strongly with the anti-AIM MoAb [25].

Next, we examined the adhesive capacity of PB and SF T cells to FN through VLA-5 FN receptors. Adhesion assays to an 80-kD proteolytic fragment of plasma FN containing the RGD sequence were performed with PB and SF T cells from seven patients. We observed that SF T cells displayed a significantly higher capacity to bind to the 80-kD FN fragment than did paired PB T cells from the same patients. Binding of T cells to FN was almost completely inhibited by anti-α5 MoAb in all cases, in PB and SF compartments (Fig. 3). Adhesion was also significantly abrogated when T cells were pre-incubated with either RGDS peptides or the blocking anti-β1 Lia.1.2 MoAb (Fig. 3) but it remained unaffected in the presence of RGES peptides (Fig. 3) or anti-CD45 MoAb (data not shown). As expected, pretreatment of T cells with anti-α4 HP2/1 MoAb did not affect their subsequent attachment to the 80-kD FN fragment, lacking the VLA-4 binding site (Fig. 3). Finally, in substrate control conditions, binding of T cells to COL I or serum seroalbumin ranged in all cases between 0 and 10%.

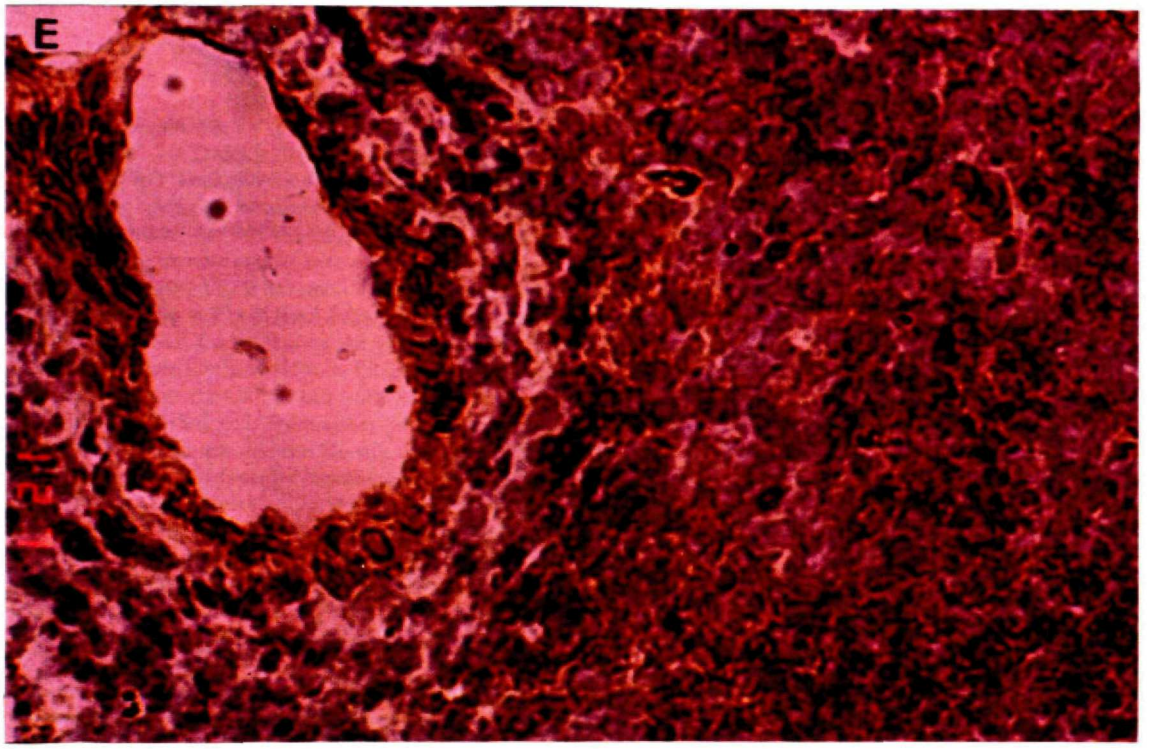
DISCUSSION

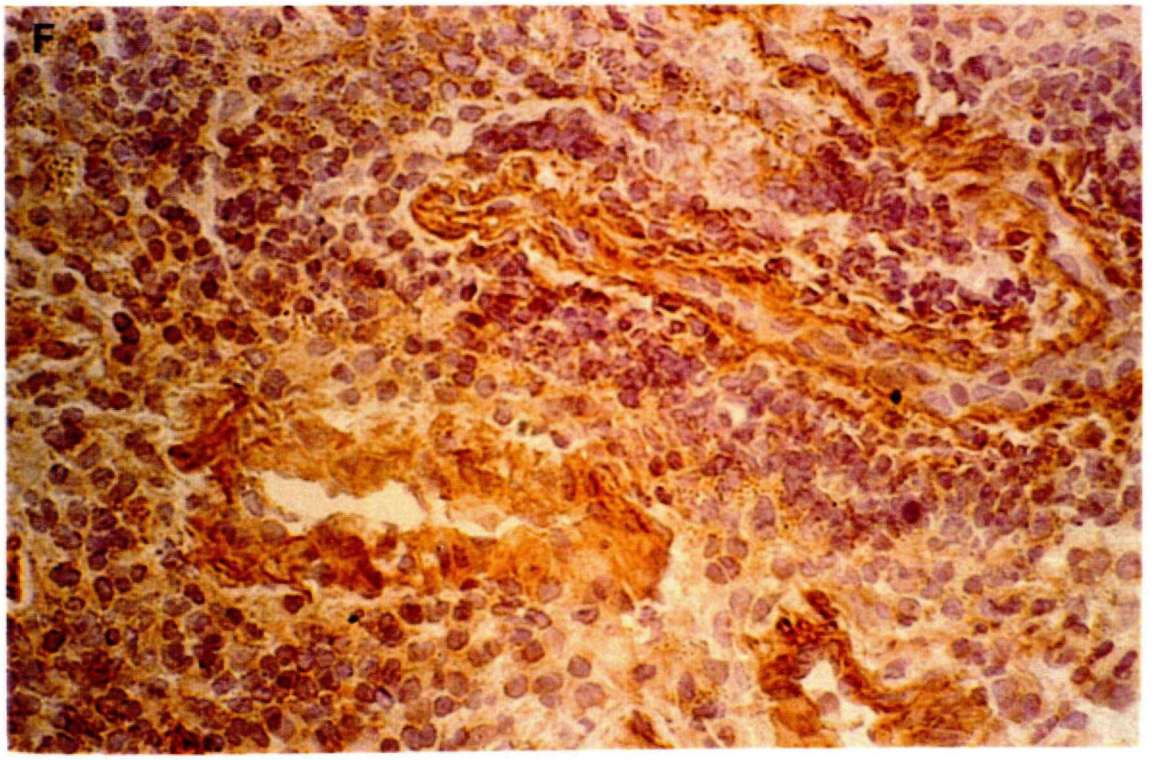
Synovial micro-environment can be considered as an intricate scenario where multiple cell–cell and cell–ECM interactions develop influenced by local release of soluble mediators. In the present study, we have shown that VLA heterodimers (α1–5, β1) are widely distributed in the SM of RA patients, emerging thus as cell surface structures potentially relevant in the interactions of immune cells with ECM proteins. VLA α1, 2, 3 and 5 subunits are expressed on synoviocytes as well as on endothelial cells, whereas the great majority of infiltrating T cells are $\alpha4^+$ and only a small percentage stains with anti-α1 or α5 MoAb. T cells in the SF compartment display a very similar pattern of VLA expression. Thus, SF T cells bear moderate expression levels of α1 and α3, raised levels of α5, and α4 was clearly over-represented. A large subset of these T cells both in SF and SM appears to be in an activation state, as shown by the expression of the activation antigen AIM (CD69) [25; this study]. This correlates with the suggested activated phenotype of SF T cells using HLA-DR and other activation markers [22–24]. An important consideration is that PB T lymphocytes, which virtually lack expression of VLA-1, 2 and 3 molecules, clearly display VLA-4 expression, although to a significant lesser extent than SF T cells. By contrast, similar expression values were detected for VLA-5 heterodimers within PB and SF from our patients. These results were consistent with those obtained in patients with other chronic rheumatic diseases.

It is well known that T lymphocytes use α5β1 (VLA-5) as a receptor for FN, through the central cell attachment domain which is contained in the 80-kD FN cell binding fragment [6]. In addition, T cells also use α4β1 (VLA-4) to bind to a totally distinct site in an RGD-independent manner [6,7]. We found that a significant proportion of SF T cells is capable of binding to the 80-kD proteolytic fragment FN, whereas only a small fraction of resting PB T cells displays this capacity. T cell binding to this fragment is specifically mediated by VLA-5 since anti-α5 MoAb and RGDS peptides abrogate T cell attachment in both compartments significantly. Otherwise, the ligand for VLA-4 is not contained in the 80-kD FN fragment and unaltered binding in the presence of anti-α4 MoAb rules out this adhesion pathway. However, the enhanced binding activity in SF (mean 45%) with respect to PB (mean 19%) in RA patients cannot be completely explained by increments in the SF VLA-5 population or by minimal changes in the surface expression of the receptors, indicating that additional mechanisms must be involved in these 80-kD FN–T cell interactions. Function of VLA-5 integrin might be modulated independently of the level of the receptor expression on the cell surface. T lymphocytes in SF and those infiltrating SM constitute an activated cell population as determined by their high expression of the activation antigen AIM. These findings suggest a possible *in vivo* upregulation of the function of VLA-5 FN receptors mediated by activation of T cells. In this context, conformational changes occurring during *in vitro* cell activation have been detected in other integrins such as LFA-1, resulting in active forms that enable receptor–ligand interactions [36]. Recently, the enhanced binding activity of three VLA members to FN (VLA-4 and VLA-5) and LN (VLA-6) has been described on PB T cells upon *in vitro* activation with phorbol esters or anti-CD3 antibodies, without detectable changes in expression [18]. Interestingly, the existence of an additional level of regulation of VLA binding



Ver texto en Figura 2 original. A) TS2/7 (anti-α1); B) P1E6 (anti-2); C) P1B5 (anti-α3); D) HP2/1 (anti-α4).





Ver texto en Figura 2 original. E) P1D6 (anti-α5); F) TS2/16 (anti-β1). Aumento: E)x1000, F)x400.

to ECM components as a result of T cell differentiation from 'naive' ($CD45RA^+RO^-$) to 'memory' T cells ($CD455RA^-RO^+$) has also been reported [18]. The latter subset exhibits a more efficient attachment to its ligand. It seems that these mechanisms act by overlapping in T cell functional behaviour, since *in vitro* studies have shown the acquisition of T cell memory marker CD45RO upon T cell activation [37]. Our phenotypic studies of T cells from different compartments show that 70–80% of SF T cells express the T cell 'memory' marker $CD45RO^+$ whereas only 30–35% in PB exhibit this phenotype [25]. Such findings correlate with results from other investigators, obtained with T cells from either patients with RA and other chronic rheumatic diseases [38–40]. Further evidence for modulation of VLA-5 function has been obtained from studies in human epidermal keratinocytes [41]. In this study, a decrease in adhesion of keratinocytes to FN was correlated with a decrease in the ability of the $\alpha5\beta1$ receptor to bind to their ligand, preceding by several hours the loss of the integrin from the cell surface [41]. It is conceivable that cells which are capable of adhering better to ECM proteins would tend to be retained in the tissue. We propose that activated SF T cells acquire functional active VLA-5 receptors upon T cell activation, which enable interaction with their ligand FN. This may represent a further mechanism of persistence at inflammatory foci of cells supposed to be previously exposed to antigen.

However, increased levels of FN have been detected in SF from RA patients compared with plasma [42,43]. This may be the consequence of a restricted and localized increase in the production of this protein by stimulated synovial cells [44–45]. The relative contribution of $\alpha5\beta1$ to anchoring T cells into synovial tissue remains unclear. The local generation and exposure of different binding sites of FN may influence the preferential migration of cells with specific functional active receptors toward gradients of higher FN fragment concentration.

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CAPITULO II

REGULACION DE LA EXPRESION Y DE LA FUNCION DE LOS RECEPTORES DE FIBRONECTINA VLA-4 EN CELULAS T ACTIVADAS DE PACIENTES CON ARTRITIS REUMATOIDE

La integrina VLA-4 (CD49d/CD29) es un receptor de superficie celular implicado en la interacción de células linfoides con la MEC y con CE. Hemos investigado la expresión y la función de los receptores de FN VLA-4 en células T del compartimento sinovial de pacientes con AR. Una gran proporción de linfocitos T del LS y de la MS expresan los antígenos de activación linfoides AIM (CD69) y gp95/85 (Fig. 1C, 1D, Tabla 1 y Fig.2B, 2C), así como un mayor número de moléculas $\alpha 4$ y $\beta 1$ en comparación con sus correspondientes de SP (Tabla 1, Fig. 1A, 1B y Fig 2A). En la MS estudios de doble tinción con inmunohistoquímica demuestran que la mayoría de las células T de los infiltrados que expresan a4 coexpresan los antígenos de activación AIM y gp95/85 (Fig. 2D y datos no mostrados, respectivamente). Además, casi todos los linfocitos en los densos infiltrados de la MS muestran el antígeno de células T de memoria CD45RO, frente a una minoría de células T vírgenes CD45RA+ (Fig 2bis). La mayoría de las células del LS (79±12%) se adhieren al fragmento de FN humana de 38kD que contiene el fragmento CS-1, ligando de VLA-4, mientras que una proporción significativamente menor de células T en SP (30±11%) muestran esta capacidad (Fig. 3). Esta adhesión está mediada específicamente por VLA-4 ya que puede ser inhibida totalmente por el AcM anti-$\alpha 4$, HP2/1 (Fig. 4). Los porcentages de células T en SP y LS capaces de adherirse a FN se correlacionan con las fracciones de células que expresan el antígeno de activación gp95/85 y el marcador de células de memoria CD45RO (Fig. 3). Por lo tanto, las células T del compartimento sinovial de AR, en su mayoría activadas y con fenotipo de células de memoria, proporcionan evidencia de la regulación *in vivo* de la expresión y función de la integrina VLA-4.

Upregulated Expression and Function of VLA-4 Fibronectin Receptors on Human Activated T Cells in Rheumatoid Arthritis

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Abstract

The VLA-4 (CD49d/CD29) integrin is a cell surface receptor involved in the interaction of lymphoid cells with both extracellular matrix (ECM) and endothelial cells. We have investigated the expression and function of VLA-4 fibronectin (FN) receptors on T cells localized in the inflammed synovium of patients with rheumatoid arthritis (RA). A high proportion of T cells in both synovial membrane (SM) and synovial fluid (SF) expressed the activation antigens AIM (CD69) and gp95/85 (Ea2) as well as an increased number of VLA-4α and β1 adhesion molecules, as compared with peripheral blood (PB) T cells from the same patients. Furthermore, the majority of these activated SF T cells were able to adhere to a 38-kD FN proteolytic fragment containing the connecting segment-1 (CS-1) specifically through VLA-4 receptors, whereas a significantly lower proportion of PB T cells displayed this capacity. Therefore, our results show that activated T cells selectively localize at sites of tissue injury in RA disease and provide evidence for the in vivo regulation of the expression and function of the VLA-4 integrin. This regulatory mechanism may enable T cells either to facilitate migration or to persist at sites of inflammation. (*J. Clin. Invest.* 1991. 88:546–552.) Key words: rheumatoid arthritis • activated T cells • fibronectin receptors

Introduction

The integrin family includes receptors for extracellular matrix (ECM)[1] components as well as receptors involved in cell–cell adhesive interactions (1–3). The β1 integrin subfamily, also known as very late activation antigen (VLA) proteins contains at least six different α chains (VLA-1–VLA-6) associated with a common β chain that function as receptors for ECM proteins such as collagen, fibronectin, and laminin (reviewed in reference 4). Recently, novel associations of α and β subunits have been found, which expand the molecular and functional repertoire of VLA integrins (4).

VLA-4 is a member of the β1 integrins that is expressed by resting lymphocytes, monocytes, as well as by most T and B cell lines (4–7). Unlike other VLA members, the VLA-4 heterodimer has been implicated in cell–cell adhesion and antibodies against human VLAα4 chain inhibit the attachment of lymphocytes to high endothelial venule cells from Peyer's patches (8, 9). A VLA-4 ligand in activated endothelial cells has been recently discovered, vascular cell adhesion molecule (VCAM-1), that belongs to the Ig gene superfamily (10, 11). In addition, VLA-4 plays a role in both heterotypic adhesion between cytolytic T lymphocytes and B cell targets (12, 13), and homotypic leukocyte aggregations triggered by anti-VLAα4 antibodies (14, 15).

Similarly to the rest of VLA proteins, VLA-4 has also been involved in cell-ECM protein interactions. Thus, VLA-4 has been demonstrated to bind, in a RGD-independent manner, to the connecting segment 1 (CS-1) of fibronectin in both T and B cell lines (10, 16, 17). Recent studies indicated that the expression of three different VLA heterodimers (VLA-4, VLA-5, and VLA-6) is regulated during T cell maturation, because higher amounts of these integrins are found on memory (CD45RO+) T cells as compared with naive (CD45RO−) T cells (18).

As described for the LFA-1 leukocyte integrin (19), the constitutive expression of VLA receptors does not necessarily imply that they are functionally active. Thus, it has been described that the in vitro activation of CD4+ T cells by phorbol esters and other stimuli induces VLA-mediated attachment to ECM proteins (18, 20).

We have addressed the question whether a regulated expression and function of the VLA-4 fibronectin receptors exists on T cells which are activated in vivo in a pathological situation. Rheumatoid arthritis (RA) constitutes a human model of chronic articular inflammatory disease, where T cells infiltrating the synovium may play an important role in the pathogenesis of this disease (21). The activation state of T lymphocytes can be investigated by means of the expression of activation molecules on the lymphocyte surface. It is well known that synovial fluid (SF) T cells are activated, because they express HLA-DR and VLA-1 antigens, although they lack expression of IL-2 and transferrin receptor molecules (22–25).

In this study, we have more precisely determined the activation state of SF T cells by studying the expression of two novel lymphocyte activation antigens: AIM (CD69), the earliest inducible molecule on T cells, which is functionally associated to the proliferative process of T cells (26), and gp95/85, an activation molecule similar in cell distribution and molecular characteristics to the previously described Ea2 activation antigen (27). The results here reported provide the first evidence for the regulation in vivo of the expression and function of the VLA-4 fibronectin receptors on activated T cells localized at sites of inflammation.

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1. *Abbreviations used in this paper:* AIM, activation inducer molecule; CS-1, connecting segment 1; ECM, extracellular matrix; FN, fibronectin; PB, peripheral blood; RA, rheumatoid arthritis; SF, synovial fluid; SM, synovial membrane; VCAM-1, vascular cell adhesion molecule; VLA, very late activation antigen.

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Methods

Subjects. 10 patients with RA according to American College of Rheumatology criteria (28) were studied. Seven patients were female and three males. Their ages ranged between 21 and 76 yr with a mean of 56.8 yr. All patients were taking nonsteroidal antiinflammatory drugs. In addition four of them were taking gold derivaties, one antimalarial drugs, one D-penicillamine, one methotrexate, and only one patient was under low doses of corticosteroid therapy. T lymphocytes from six healthy donors were used as controls.

Preparation of purified T lymphocytes. PB and SF were collected at the same time in heparinized tubes, and mononuclear cells (MNC) were isolated by Ficoll-Hypaque gradient centrifugation (Pharmacia Fine Chemicals, Uppsala, Sweden). For purification of T cells, adherent cells were removed from MNC by culturing on plastic petri dishes for 45 min at 37°C, followed by passage through a nylon-wool column. Briefly, MNC were placed on a 600-mg nylon-wool column preincubated for 30 min with RPMI 1640 (Flow Laboratories, Inc., Irvine, CA) supplemented with 10% FCS (Gibco, Grand Island, NY), 2 mM glutamine, and 50 μg/ml penicillin per streptomycin. The MNC were then incubated on the column for 45 min at 37°C. Cells were eluted with 20 ml of RPMI 1640. The purified T lymphocyte fractions contained > 90% of CD3, < 6% monocytes and < 1% B cells, as determined by expression of the CD3, Mo-2 (CD14), and B1 (CD20) antigens, respectively.

Monoclonal antibodies (mAb). The mAb directed against activation molecules in these studies were as follows: TP1/55 (anti-AIM/CD69) (26) and TP1/16 (anti-gp95/85), whose characterization will be described in detail elsewhere. Two different mAb were used to study the VLA complex: TS2/16, which recognizes the VLA β1 subunit (29), and HP2/1 directed towards the VLA-4α4 subunit (6, 14). The UCHL1 anti-CD45RO was kindly provided by Dr. P. C. Beverley (Imperial Cancer Research Foundation, London, UK).

The mAb used to assess the purity of T cell preparations were SPV-T3b (anti-CD3) (30), Bear-1 (anti-CD11b) (31), and BC-1 (anti-CD20) (32). Anti-Mo-2 (CD14) was kindly provided by Dr. J. E. de Vries (Unicet Labs, Dardilly, France). Other mAb used as control was D3/9 mAb specific for the leukocyte common antigen (T200/CD45) (33). mAb X63 (IgG1), used as a negative control, is the Ig secreted by the mouse myeloma cell line P3-X63.

Flow cytometry analysis of T cell surface activation antigens. Viable cells ($1-5 \times 10^5$) were suspended in 50-μl aliquots of PBS, pH 7.4, containing 0.5% BSA. Specific mouse mAb (1 μg in 50 μl) was added and cells were incubated for 30 min at 4°C, then washed twice and incubated with saturating amounts of fluorescein-conjugated $F(ab')_2$ goat anti-mouse Ig (Dakopatts, Copenhagen, Denmark). After three washes fluorescence was measured using an EPICS C flow cytometer (Coulter Electronics Inc., Hialeah, FL). The specific percentages of positive cells for different mAb were obtained by subtracting the number of background cells that were nonspecifically stained with the myeloma mAb × 63 control.

Immunoperoxidase staining of tissue sections. Synovial samples were obtained from surgical synovectomy in RA patients, frozen in OCT (Ames Co., Miles Laboratories, Elkhart, IN) and stored at −80°C. The tissue sections were stained by an indirect immunoperoxidase method as described (34). Briefly, 4 μm acetone fixed sections were sequentially incubated with mAb culture supernatants and peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts, Cophenhagen, Denmark); each incubation was followed by three washes. The reaction was developed with Graham-Karnovsky medium containing 0.5 mg/ml of 3-3 diaminobenzidinetetrahydrochloride and hydrogen peroxide. Sections were counterstained with Carazi's hematoxilin followed by dehydration and mounting by routine methods.

Cell attachment analysis. Human fibronectin 38 kD proteolytic fragment (from Dr. García-Pardo, Columbia University, New York) and type I collagen (Sigma Chemical Co., St. Louis, MO) were applied to 96-well flat bottom microtiter plates (Linbro; Flow Laboratories, Inc.) (40 μg/ml, 0.1 ml/well) in CO3HNa 0.1 M at 4°C overnight. Unbound binding sites were satured with RPMI 1640–1% human seroalbumin for 2 h at 37°C. Purified T cells isolated from peripheral blood and synovial fluid were added (125,000 cells/well) in 0.1 ml of RPMI 1640 and incubated at 37°C and 5% CO_2. After 30 min plates were washed with RPMI 1640 several times and examined in an inverted microscope by at least two different observers. Within each well, cells from at least three different fields were counted and referred to a nonwashed well (100% or maximum binding). Each condition was performed on duplicate. In inhibition conditions, cells were incubated for 30 min at 4°C with 25% final volume of anti-α4 HP2/1 hybridoma culture supernatant and added to wells. In control conditions, cells were incubated with RPMI 1640 alone.

Statistical analysis. Statistical analysis was performed using Student's *t* test.

Results

Previous studies have described the existence of subsets of activated T cells bearing the MHC class II and VLA-1 antigens infiltrating the synovium of patients with RA (22–24). We have determined more precisely the activation state of T cells in both SF and peripheral blood (PB) compartments by studying the expression of two novel activation antigens AIM (CD69) and gp95/85 (Ea2). A remarkable high expression of both activation molecules was observed in SF T cells as compared to the weak expression of AIM and gp95/85 by PB T cells from same patients (Table I and Fig. 1). The expression of both AIM and

Table I. Expression of VLA-4 Integrin and Activation Antigens on SF and PB T Cells from RA Patients

CD	Antigen specificity	mAb	Healthy donors (n = 6)		RA Patients (n = 10)			
			PB		PB	SF	PB	SF
			%*	*MFI*	%*		*MFI*	
CD49d	VLA-4α	HP2/1	51±15	47±11	42±18	70±13‡	45±23	62±21‖
CD29	VLA β1	TS2/16	60±18	56±16	58±15	82±10§	63±27	85±17‡
CD69	AIM	TP1/55	3±2	23±9	10±8	46±18‖	25±16	49±17‖
—	gp95/85	TP1/16	26±10	39±4	30±13	81±10‖	46±20	83±22‖
—		X63		16±6			16±11	17±11

* Values are the percent of T cells positive for each antigen (mean±SD) the specific percentages of positive cells mAb X63 control. (*MFI*) Mean fluorescence intensity (mean±SD) values are expressed in a logarithmic scale. Statistical significance: ‡ $P < 0.01$; § $P < 0.05$; ‖ $P < 0.001$.

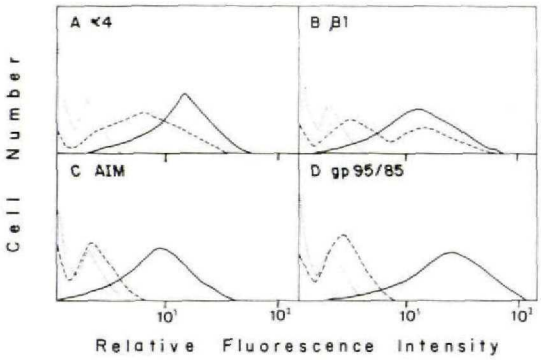


Figure 1. Expression of adhesion (VLAα4 and β1) and activation (AIM and gp95/85 molecules by synovial fluid (SF) and peripheral blood (PB) T lymphocytes from a representative patient with rheumatoid arthritis (RA). Immunofluorescence flow cytometry analysis were performed on purified SF (*solid line*) or PB (*dashed line*) T lymphocytes obtained from a patient with RA. Cells were labeled with HP2/1 mAb (anti-VLAα4), TS2/16 mAb (anti-VLA β1), TP1/55 mAb (anti-AIM), TP1/16 mAb (anti-gp95/85), and with the negative control X63 (*dotted line*). In a second step the cells were stained with a goat anti-mouse IgG-FITC.

gp95/85 activation antigens was very similar in PB T cells from either patients with RA or healthy donors (Table I). These results indicate that SF T cells represent in vivo activated T lymphocytes selectively located at the site of tissue inflammation.

To determine the possible role of the VLA-4 integrin in T cells localized at the inflammed synovium, we first examined its expression and function on T cells from both SF and PB from patients with RA. As observed in Table I, flow cytometry studies demonstrated an increased expression of both VLAα4 and β1 antigens in purified SF T cells as compared with that of purified PB T cells from same patients with RA, both in terms of percentage of positive cells and fluorescence intensity (compare flow cytometry profiles in Fig. 1). Levels of VLA-4 expression on PB T cells from patients with RA were in the same range than those displayed by PB T cells from healthy donors (Table I). However, no significant changes in the expression levels of other cell surface antigens such as CD3 or CD45 were observed between SF and PB T cells (data not shown).

Similarly to SF T cells, the infiltrating CD3+ T lymphocytes located in the SM of RA patients (data not shown), also expressed the VLA-4 integrin and the CD69 activation antigen, as assessed by immunoperoxidase staining of synovial membrane (SM) tissue sections (Fig. 2). Most of the VLA-4+ T cell aggregates infiltrating SM were in the perivascular space although some scattered VLA-4+ T cells were also observed all over SM. The majority of the AIM+ T cells in SM also coexpressed the VLA-4 antigen as demonstrated by double immunostaining with anti-AIM and anti-VLA-4 mAb. Similar results were obtained with the anti-gp95/85 mAb (data not shown).

To determine the functional capacity of VLA-4 receptors expressed by these cells, adhesion studies to the 38-kD FN fragment were performed with T cells purified from both PB and SF compartments of RA patients. The majority (79%) of SF T cells were capable to adhere to plastic plates coated with the 38 kD FN fragment, whereas only a reduced percentage (30%) of PB T cells displayed this capacity (Figs. 3 and 4). The percentages of PB T cells in RA patients that bound to 38 kD FN were in the same range to those found in PB T cells from healthy donors and correlated with the percents of lymphocytes bearing the CD45RO T cell memory marker (Fig. 3, and data not shown). Neither SF nor PB T cells bound to plates coated with collagen because they did not express detectable amounts of the VLA-2 collagen receptor (data not shown). Percentages of cell binding both in SF and PB correlated with the fractions of cells bearing the activation antigen gp95/85 and the CD45RO marker (Fig. 3).

The attachment of T cells from both PB and SF to the 38 kD FN fragment is illustrated in Fig. 4, *A* and *C*, respectively. T cell binding to this ECM component was specifically mediated through VLA-4 receptors because the anti-VLAα4 HP2/1

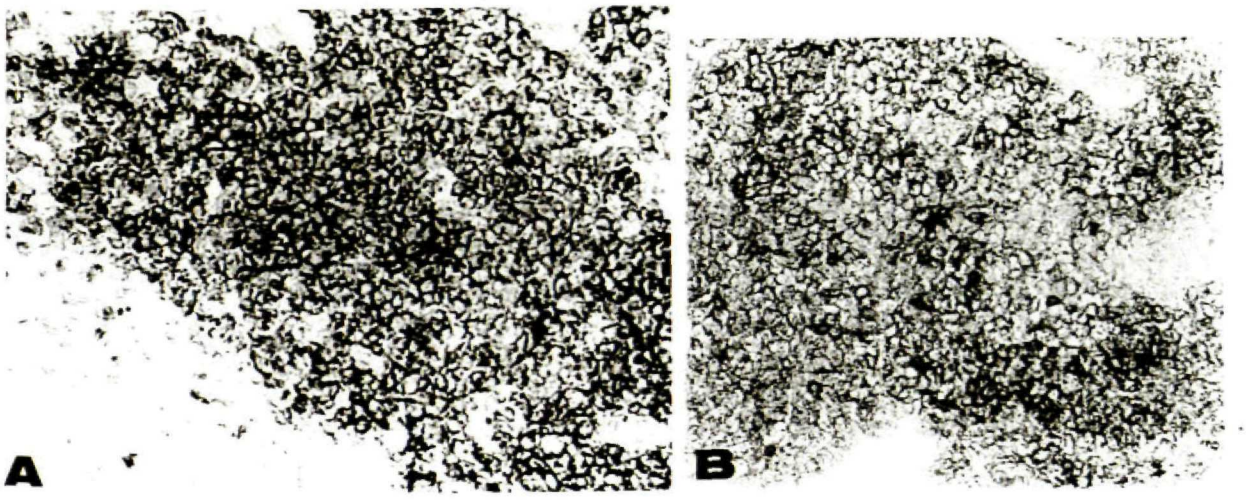


Figure 2. Immunoperoxidase staining of synovial tissue from one patient with RA, with anti-VLA-4 (HP2/1) (*A*) and anti-AIM (TP1/55) (*B*) mAb. Magnification, 400.

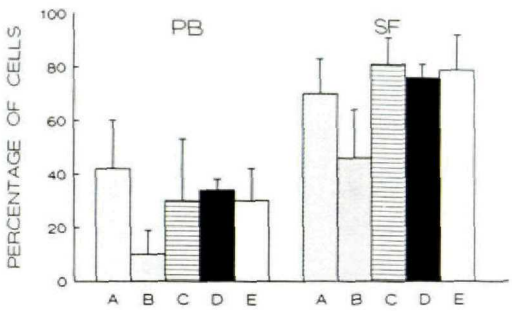


Figure 3. Comparison of the expression of VLA-4 (*A*), AIM (*B*), gp95/85 (*C*), and CD45RO (*D*) antigens and functional activity for binding to the 38-kD FN fragment (*E*) of purified T cells from both peripheral blood (*PB*) and synovial fluid (*SF*). The values of cell expression of AIM and gp95/85 activation antigens and VLA-4 integrin are the average of 10 independent experiments obtained with cells from 10 different patients with RA. The values of CD45RO cell expression and cell adhesion are means of independent experiment with both SF and PB T cells from six different patients. Percentages of FN attached cells were 79%±12 for SF T cells and 30%±11 for PB T cells.

mAb almost completely abrogated cell attachment of either SF or PB T cells (Fig. 4, *B* and *D*, respectively).

Discussion

In this study, results are reported demonstrating that T lymphocytes infiltrating both SF and SM compartments in patients with RA constitute an activated cell population bearing the CD69 and gp95/85 activation markers as well as displaying an enhanced expression and function of VLA-4 fibronectin receptor. Thus, we have found that the majority of these in vivo activated T lymphocytes possesses functionally active VLA-4 fibronectin receptors enabling their interaction with an RGD-independent cell binding site on the 38 kD proteolytic fragment of human plasma fibronectin. By contrast, a reduced fraction of T cells from the PB compartment of same patients displayed this functional capacity.

Rheumatoid synovitis is characterized by the accumulation of mononuclear cells which have a tendency to form aggregates, particularly around blood vessels (35). The cells which infiltrate the perivascular space emigrate mainly from postcapillary venules. It is now generally accepted that T cells locally infiltrating the rheumatoid synovial membrane play an important role in the pathogenesis of chronic inflammation of RA (21). A large fraction of the T cells in the synovial infiltrate and SF appears to be in an activated state, as evidenced by the fact that a majority of these cells expresses activation markers, such as the HLA-DR antigen (22, 25). Furthermore, a subset of T cells in the SF displays a low expression of the VLA-1 activation antigen, a member of the VLA integrin adhesion family, although they lack expression of IL-2 and transferrin receptor molecules (23–25). However, both HLA-DR and VLA-1 are not the more appropriate antigen markers to assess activation of T cells because they are also expressed by a wide variety of cell types at both resting and activated states (4, 35).

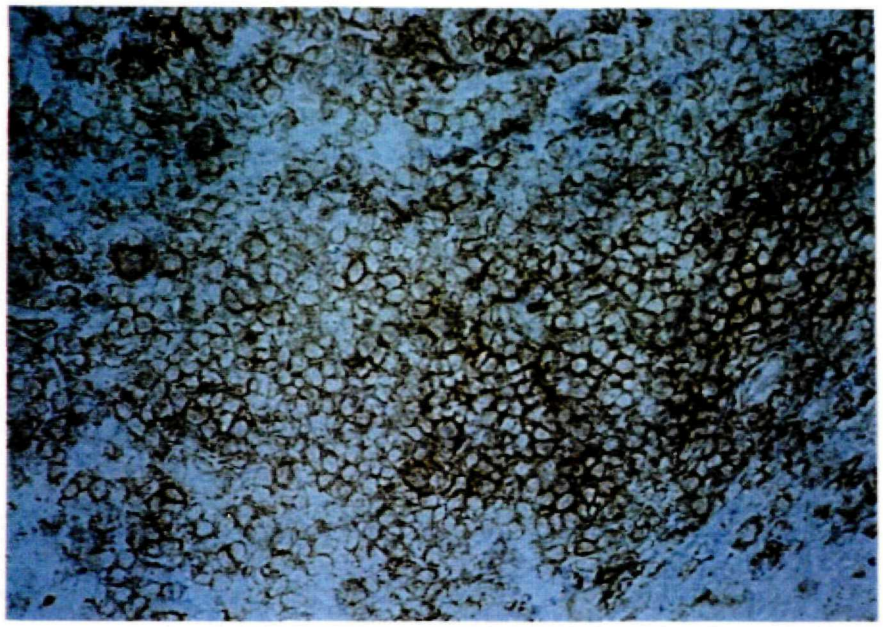
In this sense, we have previously reported the structural and functional characterization of a novel human cell activation antigen, designated as "activation inducer molecule" (AIM), recently coded as CD69, that is rapidly expressed on T and B cells upon treatment with different stimuli (26, 36). Additionally, we have isolated a mAb specific for gp95/85, a dimeric structure weakly expressed on a small fraction of resting PB T cells whose expression is strongly induced on activated lymphoid cells. The biochemical and functional characteristics of gp95/85 are similar to those of the previously described for the Ea2 activation antigen (27), although comparative studies will be required to ascertain whether both mAb are recognizing identical molecular structures.

We have found a strong expression of both AIM (CD69) and gp95/85 activation antigens on SF and SM T cells from all RA patients, compared to their absence or low expression by PB T cells from either same patients or healthy donors. These findings indicate that a large fraction of T cells infiltrating the rheumatoid SM and SF are in an activated state. Similarly, an activated T cell subset bearing the CD69 activation antigen has been recently reported in the lymphocyte infiltrate of chronic inflammatory liver diseases (37).

The percentages of RA PB T cells displaying functional capacity to adhere to 38-kD FN are within the same range of those previously described in PB T cells from healthy donors and correspond to the CD45RO+ memory T cell subset (18). The CD45RO+ PB T cells have been reported to express higher amounts of different adhesion receptor molecules than the CD45RO− naive T cells (38), correlating with their enhanced functional adhesive capacity to ECM protein components (18). We have also found that most of activated T cells in SF bear the CD45RO marker as described by other authors (39), and in agreement with in vitro studies showing the acquisition of CD45RO antigen upon T cell activation (38, 40).

The VLA-4 integrin plays a dual role as a receptor involved in both cell–cell and cell-ECM adhesive interactions through the two known ligands VCAM-1 and FN. Our data reveal that activated T cells found in SF of patients with RA expressed higher amounts of VLA-4 fibronectin receptor molecules than PB T cells, correlating with their enhanced binding activity to the 38-kD FN fragment specifically mediated by VLA-4 integrin.

There are two possible explanations for the differences in the adhesion activities found between SF and PB T cells to the 38-kD FN component. First, the overexpression of VLA-4 molecules of SF T cells, probably due to the activation process, could be responsible of the enhancement of VLA-4-mediated ECM binding. A second possibility is that cell activation process undergone by SF T cells induces functionally active forms of VLA-4 integrins by conformational changes on these molecules. This fact could reflect an in vivo situation of what it has been described in in vitro activation for different integrin molecules from the $\beta 1$, $\beta 2$, and $\beta 3$ families. In this sense, conformational changes occurring during cell activation have been suggested for gpIIb/IIIa, Mac-1 and LFA-1 integrins resulting in active forms enabling their respective receptor-ligand interactions (19, 41–45). Very recently, the enhanced binding activity of some VLA members to their respective ECM protein ligands has been described on peripheral blood CD4+ T cells upon activation with phorbol esters or CD3 and CD2 antibodies (18, 20). Finally, a combination of both explanations could also be possible. However, the nature of biochemical events responsi-



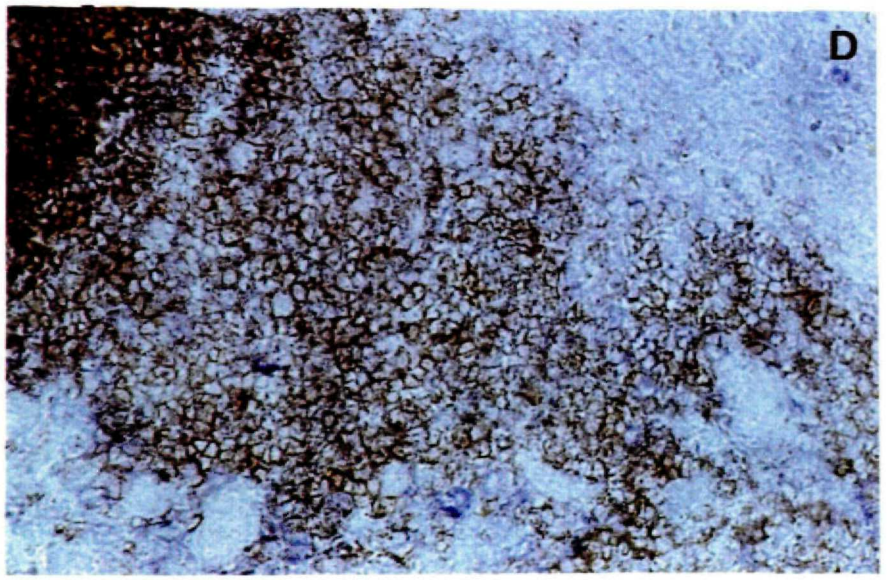


Fig. 2. Estudio inmunohistoquímico de la membrana sinovial de un paciente con Artritis Reumatoide que muestra la expresión de epítopos de activación en los linfocitos del infiltrado: C) TP1/16 (gp95/85) ; D) doble tinción con HP2/1 (anti-α4) en peroxidasa (marrón) y TP1/55 (anti-CD69) en fosfatasa alcalina (azul).

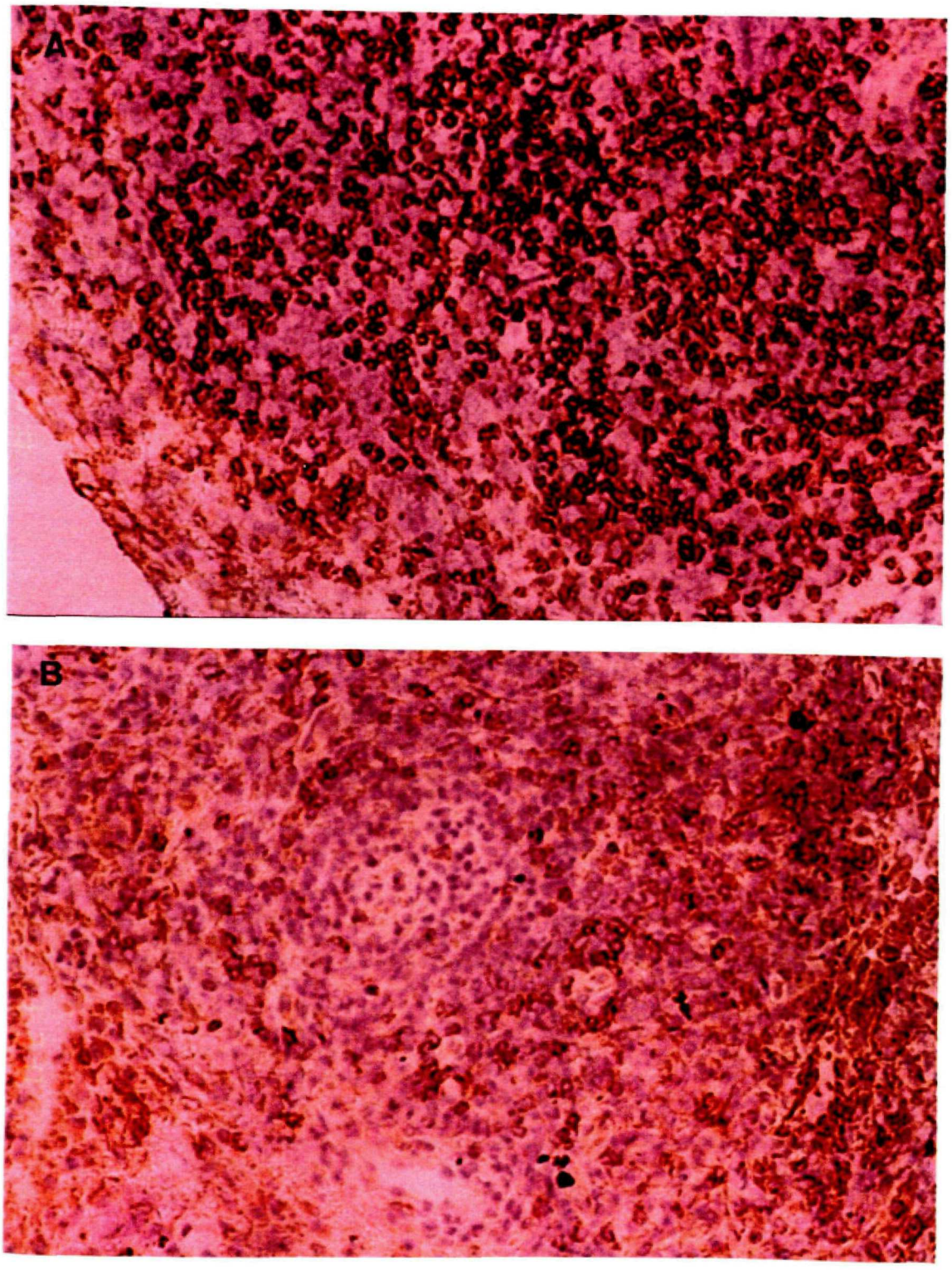


Figura 2 bis. Identificación de las células T de memoria CD45RO+, y células T vírgenes CD45RA+, en la membrana sinovial de un paciente con AR, mediante tinción con AcMs por técnica de inmunoperoxidasa. A) UCHL-1 (CD45RO); B) RP1.11 (CD45RA). Aumento: x200.

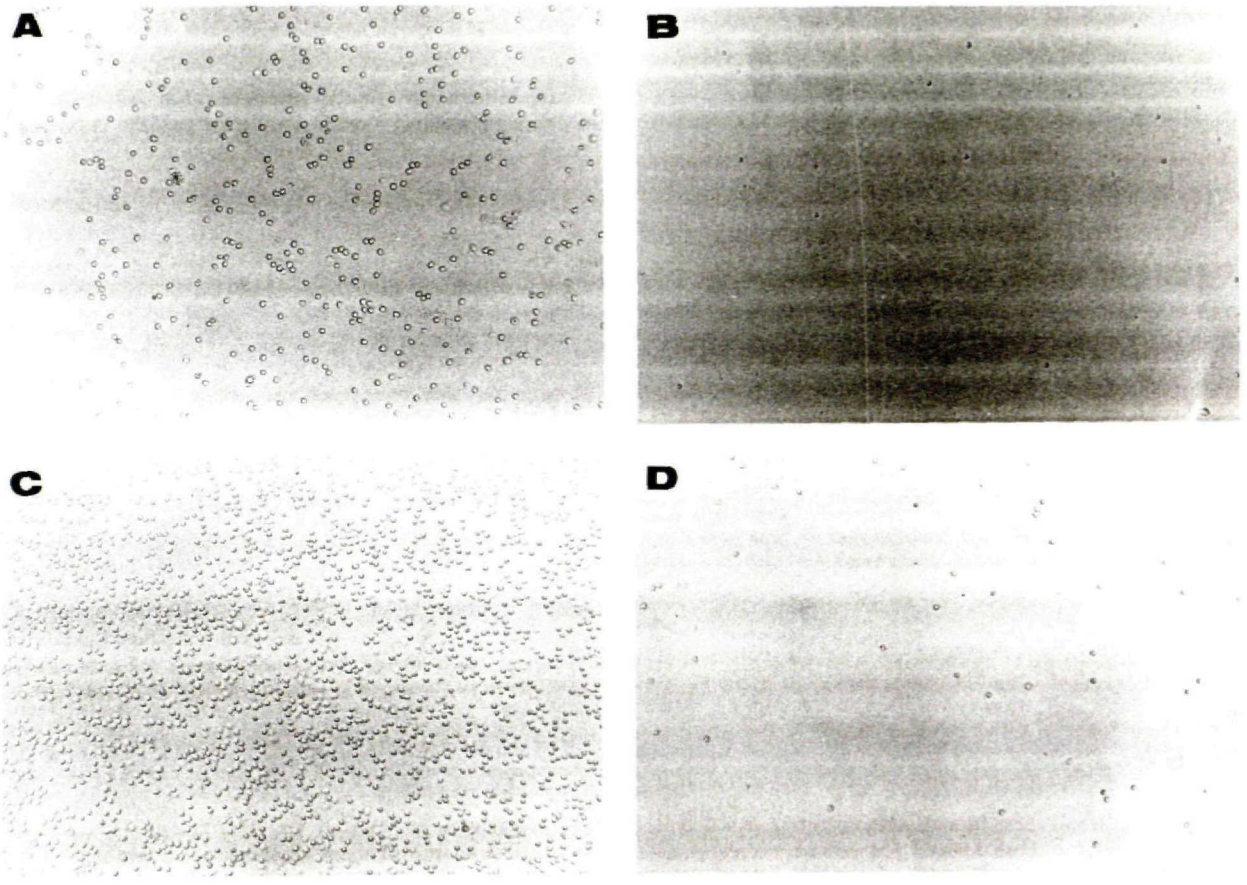


Figure 4. Binding of T cells from PB (*A, B*) and SF (*C, D*) of patients with RA to a 38-kD FN fragment either in the absence (*A, C*) or the presence (*B, D*) of anti-VLA4α HP2/1 mAb.

ble of these putative conformational changes still remains to be elucidated. It has been proposed that phosphorylation of specific residues plays a role in the regulation of the functional states of integrin molecules (46, 47).

The functional significance of the presence of in vivo activated T cells bearing functionally active VLA-4 FN receptors locally infiltrating inflamed synovium of RA patients remains uncertain. It is tempting to speculate about a role of VLA-4 in the pathogenesis of RA. Thus, it could be suggested that VLA-4 may direct the migration of these cells to sites of inflammation where a high concentration of fibronectin exists. In this sense, fibronectin has been detected in higher levels in SF from patients with RA than in plasma and other SF (48, 49). Rised levels of FN in SF from rheumatoid joints may be possibly accounted for a restricted and localized increase in the production of this protein by synovial cells at the site of the articular disease. It is therefore possible that the local production of FN within affected joints may influence the continuance of arthritis depending on the amounts synthesized by the stimulated synovial tissue (50, 51). Conceivably, these T cells have undergone an antigen-dependent activation process as deduced from their activated phenotype and have a increased number of functional FN receptors that facilitate their migration through synovial tissue towards gradients of higher fibronectin concentration. Alternatively, the adherence of cells to FN through VLA receptors could represent a mechanism of persistency of T cells at sites of inflammation. Whether a similar regulatory mechanism in operating in other VLA receptors also expressed by T cells such as VLA-5 and VLA-6 and its possible role in the RA pathogenesis remains to be elucidated.

The VLA-4 molecule may play an additional role in lymphocyte-endothelial cell interactions through its ligand VCAM-1, (10, 52). The relevance of VLA-4 in the lymphocyte entry to the inflammed synovium still remains undetermined. In the early stages of RA, coincident with the neovascularization of the SM, circulating lymphocytes adhere to endothelium in postcapillary synovial venules. T cells bearing CD45RO marker have been reported to adhere better to endothelial cells than CD45RO- cells (53) and thus, gain access more easily to the synovial membrane (54). Further studies are required to determine whether a functional regulation of VLA-4/VCAM-1 interaction may exist on SF T cells, expressing the CD45RO marker, in a similar fashion to that here described for VLA-4/FN. Cell binding assays of both SF and PB T cells from patients with RA to VCAM-1 transfectant cells as well as to endothelial cells from RA synovial tissue will be required to ascertain the role of VLA-4 in the lymphoid traffick to inflamed synovium in RA disease.

Acknowledgments

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CAPITULO III

ADHESION AUMENTADA DE LINFOCITOS T DE COMPARTIMENTOS SINOVIALES OBTENIDOS DE ARTRITIS REUMATOIDE A LAS MOLECULAS DE ADHESION ENDOTELIALES ELAM-1 Y VCAM-1

La infiltración de la MS por células mononucleares, en su mayoría células T, es una característica histopatológica típica de la AR. La entrada de linfocitos T al tejido sinovial desde el torrente sanguíneo debe estar mediada por moléculas inducidas en el endotelio por mediadores inflamatorios. En este apartado se ha investigado la adhesión de células T, obtenidas de compartimentos sinoviales de pacientes con Artritis Reumatoide, a dos ligandos endoteliales: 1) ELAM-1, una selectina capaz de funcionar como ligando vascular de células T, y 2) VCAM-1, el ligando celular de VLA-4. Como ya se había demostrado previamente (Capitulo II de Resultados), la mayoría de las células T obtenidas de LS y MS, presentan un fenotipo activado (AIM+) y de células T de memoria (CD45RO+), y una mayor expresión de los heterodímeros $\alpha 4$ y $\beta 1$ respecto a células T de SP de los mismos enfermos o de controles sanos (Tabla I). Nuestros resultados muestran que una subpoblación significativamente mayor de linfocitos T en LS Y MS son capaces de adherirse específicamente a ELAM-1 frente a la mínima población de células T en SP de enfermos o controles que muestra adhesión (Fig. 1, Fig. 2A y 2B). La mayoría de las células T de LS y MS se adhieren a VCAM-1 frente a la baja adhesión que muestran las células de SP (Fig. 2C y 2D y Fig. 3). La inhibición con AcM anti-VCAM-1 y anti-$\alpha 4$ muestra que esta adhesión es específica y mediada por la integrina VLA-4 (Fig. 3). La activación *in vitro* con ésteres de forbol o AcM anti-CD3 aumenta llamativamente la adhesión de las células T de SP a los dos ligandos de VLA-4, VCAM-1 y FN 38kD, mientras que la importante adhesión basal de las células del LS, activadas *in vivo*, apenas se modifica (Fig 4). Sin embargo cuando se hacen ensayos de adhesión con diferentes dosis de ambos ligandos, las células T del LS, aumentan su adhesión basal al ser estimuladas con PMA (Fig 5)., lo que sugiere la existencia de varios niveles de afinidad de VLA-4 por sus dos ligandos.

Tanto las células T de SP como de LS mostraron intensas respuestas proliferativas cuando se cultivaban con FN 38kD o VCAM-1 combinadas con dosis submitogénicas de AcM anti-CD3 (Tabla II).

Increased Binding of Synovial T Lymphocytes from Rheumatoid Arthritis to Endothelial-Leukocyte Adhesion Molecule-1 (ELAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1)

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Abstract

The infiltration of the synovial membrane (SM) by mononuclear cells, mostly T cells, is a typical histopathological feature associated with rheumatoid arthritis (RA). The entry of T lymphocytes into the SM is believed to be mediated by a number of molecules in the endothelium that are induced in response to a series of inflammatory mediators. In this study, we have investigated the adhesion of synovial T cells from RA patients to two endothelial ligands: endothelial-leukocyte adhesion molecule-1 (ELAM-1), the only selectin known to function as a vascular addressin for T cells, and vascular cell adhesion molecule-1 (VCAM-1), the cellular ligand of VLA-4. Our results clearly demonstrate that synovial T cells isolated from both SM and synovial fluid (SF), bearing an activated and memory phenotype, displayed an enhanced capacity to interact with these two endothelial molecules as compared with T cells from peripheral blood (PB) either of the same RA patients or healthy donors. A further enhancement of VLA-4-mediated T cell binding to VCAM-1 and fibronectin could be observed when already in vivo-activated synovial T cells were stimulated in vitro with phorbol esters, suggesting the existence of several cellular affinity levels for both very late activation-4 (VLA-4) ligands. Moreover, both PB and synovial T cells from RA patients exhibited strong proliferative responses when they were cultured with either fibronectin or VCAM-1 in combination with submitogenic doses of anti-CD3 mAb. This increased endothelial binding ability of synovial T lymphocytes together with their proliferation in response to the interaction with VCAM-1 and fibronectin may represent important mechanisms in the regulation of T cell penetration and persistence in the chronically inflamed SM of RA. (*J. Clin. Invest.* 1992. 89:1445–1452.)

Key words: rheumatoid arthritis • T cell adhesion • endothelium • selectins • integrins

Introduction

RA constitutes one of the paradigms of chronic inflammatory processes in humans. T cells emigrating from peripheral blood (PB)[1] and infiltrating the RA synovial membrane (SM) are believed to play an important role in the pathogenesis of the disease (1–4). The entry of PB T lymphocytes to the SM requires, as a first step, the binding of T cells to the endothelium (reviewed in refs. 2 and 3). A wide array of adhesion molecules has been described to be involved in this interaction (5–8). Recent reports indicate that the initial leukocyte interaction with endothelium is mediated by CD62 (PADGEM/GMP140) and endothelial-leukocyte adhesion molecule-1 (ELAM-1) molecules that belong to a newly described group of structurally related cell adhesion receptors, termed selectins (9–14), which also include the MEL-14/LAM-1 homing receptor (15). In myeloid cells, certain carbohydrate determinants, Lewis × and sialylated Lewis × epitopes, have been described as ligands for CD62 and ELAM-1 (16–20). A T cell subset has been demonstrated to bind to the endothelial ELAM-1 molecule; however, the T cell counter receptor for this selectin remains unidentified (21–23).

Once the leukocyte selectin-mediated binding to endothelial cells (EC) has taken place, the leukocyte integrins and their counter receptors in EC participate in the subsequent shear-resistant attachment to endothelium, and in the penetration into the inflammed tissue (11–14). Integrins, comprising a group of $\alpha\beta$ heterodimers involved in both cell–cell contacts and cell-extracellular matrix (ECM) interactions (7, 8), have also been implicated in lymphocyte binding to endothelium. While lymphocyte function associated antigen-1 (LFA-1) interacts with ICAM-1 and ICAM-2 (24–26), very late antigen-4 (VLA-4) integrin binds to other endothelial ligand known as vascular cell adhesion molecule-1 (VCAM-1)/inducible cell adhesion molecule 110 (INCAM-110) (27, 28). VLA-4 is the only integrin that, in addition to its involvement in leukocyte–endothelial adhesion, has also been implicated in the attachment to the ECM component fibronectin (FN) (29, 30).

The pathogenesis of RA lesions remains controversial. Some reports show the increment of an activated circulating T cell population in RA (31) which should, therefore, be able to migrate and bind to EC in SM. Other authors emphasize the role of local factors within the SM itself, especially infiltrating T lymphocytes (reviewed in references 2 and 4). In any case, an upregulated expression of some adhesion molecules (VLA-1 and VLA-4), as well as the expression of a memory

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1. *Abbreviations used in this paper:* EC, endothelial cells; ECM, extracellular matrix; ELAM-1, endothelial-leukocyte adhesion molecule-1; FN, fibronectin; FN38, 38-kD proteolytic FN; MFI, mean fluorescence intensity; MNC, mononuclear cells; PB, peripheral blood; PMA, phorbol 12 myristate 13 acetate; rsELAM-1, recombinant soluble ELAM-1; rsVCAM-1, recombinant soluble VCAM-1; SF, synovial fluid; SM, synovial membrane, VLA, very late antigens.

(CD45RO+) and activated (HLA-DR, CD69/AIM) phenotype by synovial T cells, have been recently documented (32–37).

In this study, we have investigated the binding of T lymphocytes isolated from the three compartments (peripheral blood, synovial membrane, and synovial fluid [SF]) of RA patients to two adhesion molecules specifically expressed by activated endothelial cells: ELAM-1 and VCAM-1. Our results show an upregulated T cell adhesiveness to both EC ligands, as well as an increased attachment to FN. The significance of these phenomena are discussed in the context of the RA pathogenesis.

Methods

Patients. 13 patients diagnosed as having RA according to American College of Rheumatology criteria (38) were studied. SF and PB were collected from all of them. Synovial tissue was obtained during synovectomy in two of these RA patients undergoing knee surgery. Their mean age was 55.1 yr (ranging from 30 to 60 yr), and the duration of disease ranged from 2 mo to 40 yr, with a mean of 12 yr of evolution. Four patients were taking nonsteroidal antinflammatory drugs, four were on prednisone (< 20 mg/d), two patients were treated with methotrexate, three were treated with gold salts, one with antimalarial compounds, and one with D-penicillamine.

Monoclonal antibodies. CD3 SPV-T3b mAb was kindly provided by Dr. De Vries (UNICET Labs, Dardilly, France) (39). HP2/1 is directed against α4 chain of VLA-4 (40). TS2/16 mAb recognizes the β1 common chain of VLA family, and was previously characterized (41). Anti-VCAM-1 4B9 mAb (42) was the generous gift of Dr. Harlan (University of Washington, Seattle, WA). BB11 mAb recognizes ELAM-1, as previously described (43). TP1/55 and TP1/8 mAb, recognizing AIM/CD69, and D3/9 mAb, directed against CD45, were obtained in our laboratory (44, 45). Anti-CD45RO UCHL1 mAb was kindly supplied by Dr. Beverley (Imperial Cancer Res. Fund, London, UK) (46). CSLEX1 mAb that recognizes sialylated Lewis x antigen was generously provided by Dr. Hardy (University of California, Los Angeles, CA.) (47).

Cell isolation and culturing. Peripheral blood and SF samples were collected at the same time on heparinized tubes. T cells (> 90% CD3+ and < 2% CD11b+ and CD19+) were purified from mononuclear cells (MNC) obtained after Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation and removal of adherent cells on plastics flasks (Costar Corp., Cambridge, MA). Purification was achieved by passage through nylon wool columns (DuPont/NEN, Boston, MA). Peripheral blood T cells from five voluntary healthy donors were obtained as described for RA patients' T cells.

Synovial membranes (SM) obtained at the time of surgery from RA patients were treated for 2 h at 37°C with 2 mg/ml of type P collagenase (Boehringer Manheim Diagnostics, GmbH, Germany) in serum-free RPMI 1640 culture medium (Flow Laboratories, Inc., Irvine, Scotland). Cells were maintained overnight at 37°C in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomicin (all from Flow Laboratories Inc.) (complete medium). After overnight incubation, nonadherent cells were subjected to a Lymphoprep™ gradient centrifugation, and MNC so obtained passed through nylon–wool columns. This T cell-enriched population was > 90% CD3+ and < 2% of CD19+, CD11b, or CD14+.

Reagents. Phorbol 12 myristate 13 acetate (PMA) and F(ab')2 fragments of sheep anti–mouse IgG antiserum were purchased from Sigma Chemical Co. (St. Louis, MO).

Flow cytometry analysis. T cells were incubated with hybridoma culture supernatants for 30 min at 4°C. After two washes with cold PBS, the cells were stained with a goat anti–mouse F(ab')2 fragment-FITC (Dakopatts, Copenhagen, Denmark), followed by another two washes with cold PBS. Single color fluorescence was analyzed by flow cytometry, using a EPICS-C (Coulter, Corp., Hialeah, FL). The final percentage of positive cells was obtained by subtracting the values of negative control ×63 from those obtained with every specific marker.

Cell attachment assays. Recombinant soluble VCAM-1 (rsVCAM-1) and ELAM-1 (rsELAM-1) were purified by immunoaffinity chromatography from conditioned medium of Chinese hamster ovary (CHO) cells stably transfected with a truncated cDNA for VCAM-1 and ELAM-1, respectively (23, 48). A 38-kD proteolytic fibronectin (FN38) fragment was kindly supplied by Dr. A. García-Pardo (Centro de Investigaciones Biológicas, CSIC, Madrid), and obtained as described (29). Type I collagen was purchased from Sigma Chemical Co.

Adhesion assays were performed as previously described (37). Briefly, 96-well microtiter EIA II Linbro plates (Flow Laboratory Inc.) were coated overnight at 4°C with 50 μl of different proteins (40 μg/ml of FN38 or collagen, and 10 μg/ml of rsVCAM-1 or rsELAM-1) dissolved in CO3HNa 0.1 M. Unbound sites were saturated with RPMI 1640-1% HSA for 2 h at 37°C. Thereafter, plates were gently washed with RPMI 1640 and 1.5×10^5 cells in 100 μl were added to wells and incubated for 30 min at 37°C. Then, plates were washed three times and examined in an inverted microscope by at least two independent observers. Each condition was performed in duplicate. Within each well, cells from at least three different fields were counted. The number of cells counted in a nonwashed well was considered as maximum binding. In inhibition assays, cells were incubated for 30 min at 4°C with 25% final volume of hybridoma culture supernatants of anti-α4 HP2/1 or anti-CD45 D3/9 mAb, or with purified anti-VCAM-1 4B9 or anti-ELAM-1 BB11 mAb at 10 μg/ml.

Proliferation assays. Flat-bottomed 96-well culture plates (Costar Corp.) were coated with 50 μl of anti-CD3 SPV T3b mAb at 2 μg/ml (100 ng/well) in PBS pH 8.0. Upon 4 h incubation at 37°C, plates were gently washed with RPMI 1640, and 50 μl of either FN38 fragment or rsVCAM-1 at 10 μg/ml (500 ng/well) was added and reincubated for another 4 h at 37°C. Plates were gently washed again with RPMI 1640. Purified PB and SF T lymphocytes at 0.75×10^6/ml were added in 100 μl of RPMI 1640-10% FCS to wells completing, to reach 200 μl, with complete medium or other stimuli (PMA at 2 ng/ml or anti–CD69 TP1/8 mAb at 2 μg/ml). Plates were incubated for 72 h in a 37°C and 5% CO_2 atmosphere. Cell proliferation was estimated by [^{3}H]TdR (DuPont) incorporation during the last 18 h of culture. Cells were harvested in a cell harvester (Skatron, Lier, Norway), and the radioactivity measured in a liquid scintillation β counter (Kontron Analytical, Zurich, Switzerland).

Statistical analysis. Statistical analysis was performed using Student's *t* test in the SIGMA-TM database program.

Results

Enhanced binding to the ELAM-1 selectin by a synovial T cell subset. It has recently been described that a subset of normal memory CD45RO+ T cells, as well as some T cells isolated from chronic inflammatory lesions of skin, interact with ELAM-1 in an activation-independent manner (21–23, 49). The expression of ELAM-1 on EC from a variety of inflammatory processes, including RA, has been documented (49, 50). Therefore, we investigated the interaction of synovial T cells, most of them expressing a memory CD45RO+ phenotype (Table I), with rsELAM-1. Synovial T cells derived from both SM and SF displayed a significantly higher binding (approximately twofold) to rsELAM-1, as compared with either patients' and healthy donors' PB T cells (Fig. 1). The typical pattern of enhanced attachment to rsELAM-1 by PB and SF T cells from one RA patient is illustrated in Fig. 2, *A* and *B*.

The specificity of this binding was confirmed by its inhibition by anti-ELAM-1 BB11 mAb, and the lack of effect of anti-CD45 D3/9 mAb (Fig. 1 and data not shown). No significant binding to collagen or albumin was observed (see below and data not shown). No expression of the sialylated Lewis × carbohydrate determinant was demonstrable by flow cytome-

Table I. Expression of VLA-4, CD69/AIM and CD45RO Antigens by T Cells from PB, SM, and SF of RA Patients, and from PB of Healthy Donors

CD	Antigen specificity	mAb	Healthy donors		RA patients					
			PB		PB	SM	SF	PB	SM	SF
			%	*MFI*		%*			*MFI*	
CD49d	α4 (VLA-α4)	HP2/1	53	46	43	71	73	44	71	60
CD29	β1 (VLA-β)	TS2/16	65	56	56	96	83	63	100	85
CD69	AIM	TP1/55	2	23	8	68	50	28	73	58
CD45RO	LCA	UCHL-1	41	39	37	65	74	40	95	90
—	—	×63		16				16	20	16

* The expression of both α4 and β1 chains of VLA-4 integrin, the CD69/AIM activation antigen, and the CD45RO memory marker, was analyzed by immunofluorescence flow cytometry in T cells from PB of healthy donors, and T cells isolated from different compartments of RA patients. Values represent the percentage of positive cells and were calculated as described in Methods. Mean fluorescence intensity (MFI) values are expressed in a logarithmic scale.

try analysis on either PB or SF T cells (data not shown), indicating that these glycosylated epitopes, present on myeloid cells (18, 19), are not incriminated in the observed T cell interactions with ELAM-1.

Most synovial T cells from rheumatoid arthritis patients bind to VCAM-1. The expression of VCAM-1 has been detected on EC, synovial macrophages, and in the lining layer macrophages of RA synovial tissue (50–51). Very recently, in vitro activation has been described to induce a strong enhancement in T cell binding to VCAM-1 (52). Moreover, a higher expression of both VLA-4 chains, as well as some activation antigens (CD69/AIM, HLA-DR), could be noticed in synovial T cells isolated from both SM and SF, as compared with PB (Table I). Therefore, we explored the binding of in vivo-activated synovial T cells from RA patients, as well as their PB T cells, to rsVCAM-1. The majority of synovial T cells from both SF and SM were able to bind to rsVCAM-1, in contrast to the low binding typical for PB T lymphocytes from those patients, as well as healthy donors (Fig. 3). The differential capacity of SF and PB RA T cells in adhesion to rsVCAM-1 could be evidenced in Fig. 2, *C* and *D*.

This upregulated T cell attachment to rsVCAM-1 was specifically mediated by VLA-4 integrin, since it was virtually abrogated by both anti-α4 HP2/1 mAb and anti-VCAM-1 4B9 mAb, but not by the unrelated anti-CD45 D3/9 mAb (Fig. 3).

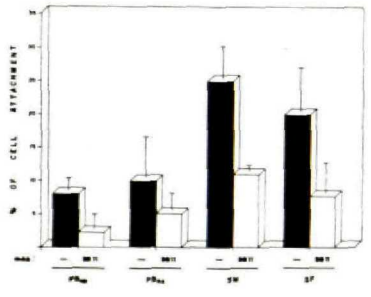

Figure 1. Binding to rsELAM-1 by T cells from PB of healthy donors, and from different compartments of RA patients. (PB_{HD}) T cells from PB of healthy donors; (PB_{RA}) T cells from PB of RA patients; (SM) T cells isolated from SM of RA patients; (SF) T cells isolated from the SF of RA patients. Adhesion assays were performed as described in Methods, either in the absence or presence of anti-ELAM-1 BB11 mAb. All the inhibitions with the BB11 mAb were significants with the next values: (PB_{HD}) $P < 0.001$; (PB_{RA}) $P < 0.01$; (SM) $P < 0.01$; (SF) $P < 0.01$. The enhanced binding to rsELAM-1 exhibited by synovial T cells, as compared with PB_{RA}, reached statistical significance with the next values: (SM/PB_{RA}) $P < 0.01$; and (SF/PB_{RA}) $P < 0.01$.

Regulation of PB and synovial T cell binding to VCAM-1 and fibronectin by in vitro activation. VLA-4-mediated adhesion to both FN and VCAM-1 ligands has been reported to be upregulated upon in vitro cell activation (52–54). This enhancement in VLA-4 adhesive properties seems to be due to a qualitative change undergone by this integrin during cellular activation. Previously, we have also documented an enhanced binding to FN by synovial T cells, as compared with PB T cells in RA patients (37).

We next investigated whether PB and SF T cell attachment to both VLA-4 ligands, VCAM-1, and a 38 kD FN fragment containing the CS-1 domain (FN38) (29), could be further upregulated by in vitro activation. As expected, treatment for 30 min with phorbol esters or crosslinked CD3 mAb enhances PB T cell adhesiveness to both VLA-4 ligands (Fig. 4). By contrast, no significant enhancement in SF T cell binding to VCAM-1 and FN38 was observed upon PMA and CD3 treatment (Fig. 4). The lack of a further regulation of SF T cell binding to VCAM-1 and FN38 by in vitro stimulation could be due to their constitutive in vivo-activated state, making them unresponsive to an additional stimulus. Alternatively, one could argue that the ligand concentrations used in this study were high enough to completely saturate their receptors, thus allowing for maximal binding reactions. If this were the case, then one would not detect differences on binding probably reflecting the existence of multiple levels of cellular affinity for FN38 and VCAM-1. To clarify this point, we assayed PMA-treated and untreated synovial and PB T cells for binding to FN38 and rsVCAM-1 applied at different doses. As illustrated in Fig. 5, an increase in cell attachment of PMA-treated versus untreated SF T lymphocytes was observed at FN38 and rsVCAM-1 concentrations below 15 μg/ml and 1 μg/ml, respectively. Thus, in vivo-activated SF T cells exhibited a higher capacity than PB T cells to interact with both VLA-4 ligands, but in vitro activation increased the adhesiveness even more, at least partially reflecting qualitative changes of this integrin (37).

Induction of proliferative signals through VLA-4 in T cells from RA patients. The interaction of T cells with several ECM proteins such as fibronectin, laminin, or collagen has been reported to trigger mitogenic signals through different VLA

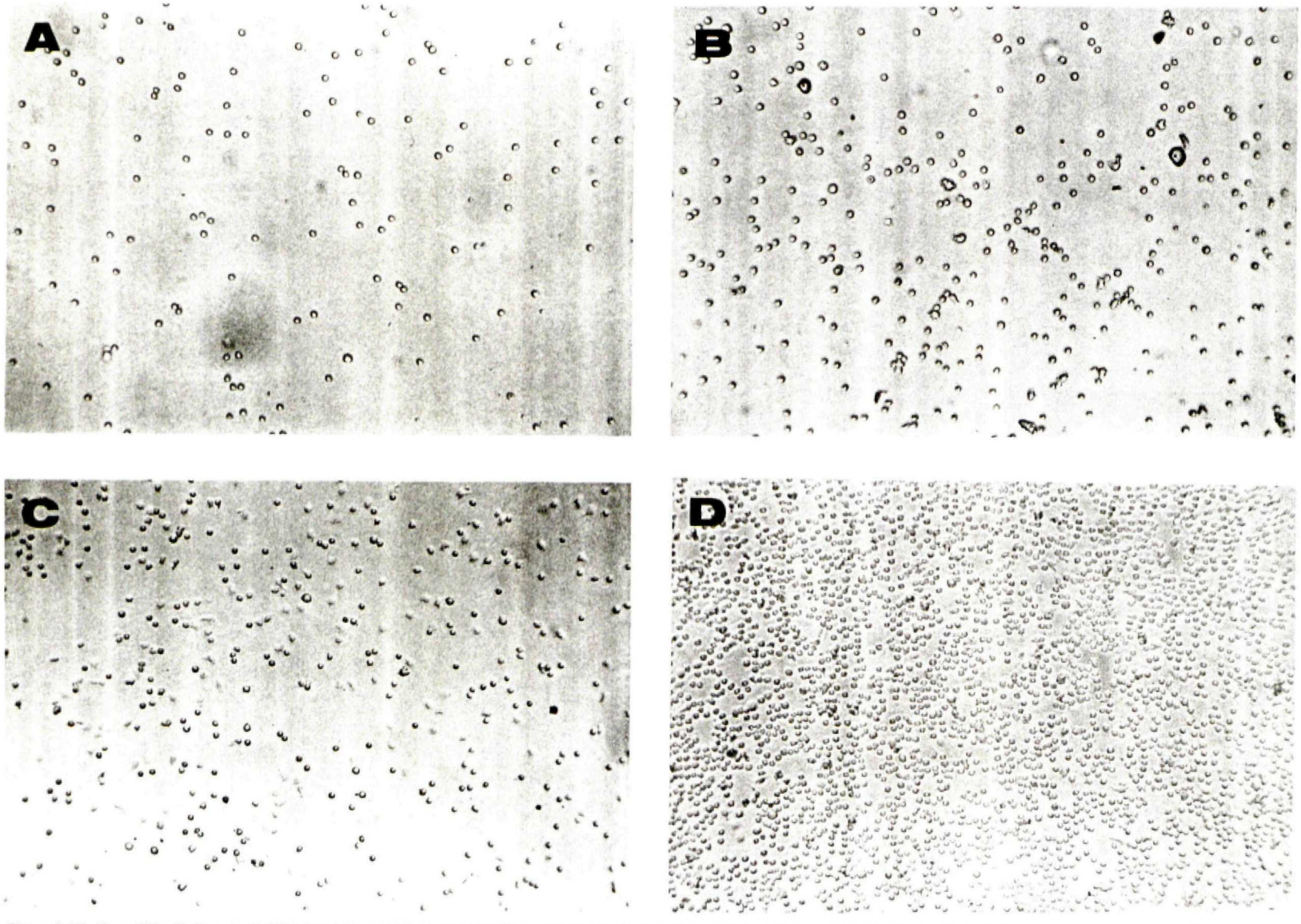

Figure 2. Binding of T cells from both PB (*A* and *C*) and SF (*B* and *D*) of RA patients to either rsELAM-1 (*A* and *B*) or rsVCAM-1 (*C* and *D*). Adhesion assays were performed as described under Methods section. ×250.

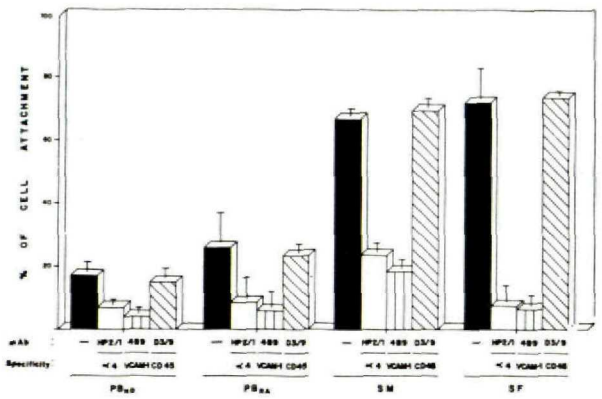

Figure 3. Binding to rsVCAM-1 by T cells from PB of healthy donors and from different compartments of RA patients. (PB_{HD}) T cells from PB of healthy donors; (PB_{RA}) T cells from PB of RA patients; (*SM*) T cells from SM of RA patients; (*SF*) T cells from SF of RA patients. Adhesion assays were performed as described in Methods, either in the absence of any mAb or in the presence of anti-VLA-α4, anti-VCAM-1, or anti-CD45 mAb. The inhibition of T cell binding to rsVCAM-1 by anti-α4 HP2/1 mAb was significant with the next values: (PB_{HD}) $P < 0.01$; (PB_{RA}) $P < 0.05$; (*SM*) $P < 0.01$; (*SF*) $P < 0.001$. The inhibition of T cell binding to rsVCAM-1 by anti-VCAM-1 4B9 mAb reached statistical significance with the next values: (PB_{HD}) $P < 0.01$; (PB_{RA}) $P < 0.05$; (*SM*) $P < 0.01$; (*SF*) $P < 0.001$. The enhancement of synovial T cell binding to rsVCAM-1, as compared with PB_{RA}, was significant with the next values: (SM/PB_{RA}) $P < 0.001$ and (SF/PB_{RA}) $P < 0.001$.

members (55–59). Immobilized FN conveys proliferative signals to CD3-activated T cells mainly through VLA-5, but also by VLA-4 (55–58).

To elucidate the response of PB and SF T cells from RA patients to both VLA-4 ligands, proliferative assays with T cells activated with anti-CD3 mAb in conjunction with either FN38 or rsVCAM-1 were carried out. Interestingly, VCAM-1 supported strong comitogenic responses with CD3 mAb in both

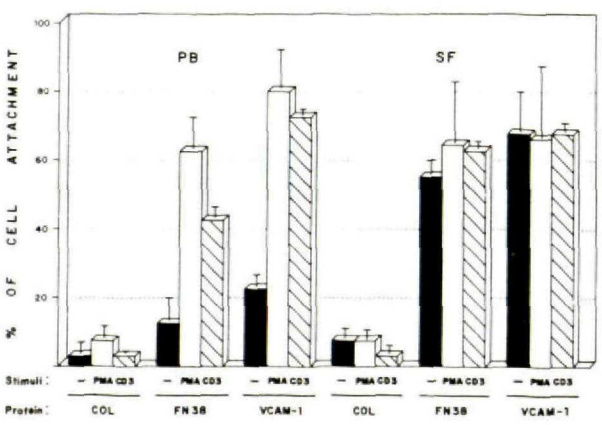

Figure 4. Binding of T cells from both PB and SF to either type I collagen (COL), a FN38 fragment, or rsVCAM-1. T cells were either untreated, treated for 30 min with PMA 50 ng/ml, or treated for 30 min with soluble anti-CD3 SPV-T3b mAb (10 μg/ml) plus anti-mouse IgG antiserum (1 μg/ml). Adhesion assays were performed as described in Methods.

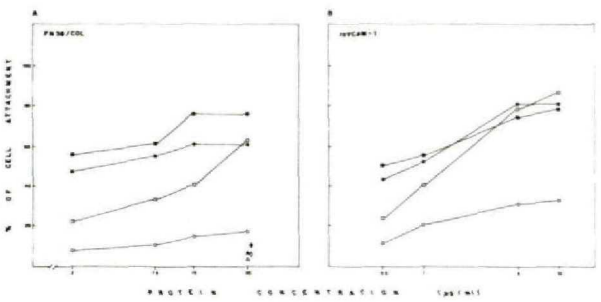

Figure 5. Binding of T cells from PB (○, ●) or SF (□, ■) of RA patients to either FN38 (*A*) or rsVCAM-1 (*B*) applied to plates at different doses. Binding to type I collagen (*COL*) by both PB (△, ▲) or SF (◇, ◆) is also represented. T cells were either untreated (*open symbols*) or treated for 30 min with PMA 50 ng/ml (*closed symbols*).

PB and SF T cells (Table II). FN38 also increased the proliferation of CD3-treated T cells, although in a lower extent than VCAM-1, which is in agreement with previous reports demonstrating that VLA-5 is the major FN receptor implicated in T cell proliferation triggered by this ECM protein (55–58). Neither rsVCAM-1 nor FN38 induced comitogenic effects in combination with PMA, although this phorbol ester showed to be strongly comitogenic with anti-CD69 TP1/8 mAb (data not shown).

Discussion

The results reported herewith demonstrate that synovial T cells from RA patients exhibit a higher capacity to interact with VCAM-1, as compared with PB T cells from the same patients. Furthermore, synovial T cells show an enhanced binding, about twofold, to ELAM-1 which could probably reflect the activity of a T cell subset. The increased adhesiveness of synovial T cells to their endothelial ligands can thus reflect pathologic phenomena occurring in the inflamed tissue of patients with RA, and, therefore, extend our current understanding of the pathophysiology of this disease. Although the patients stud-

Table II. Comitogenic Effects of FN38 and VCAM-1 in CD3 mAb-stimulated T Cells from PB and SF RA Patients

	Experiment 1		Experiment 2	
	Medium	CD3 mAb	Medium	CD3 mAb
A. PB				
Medium	0.23±0.15	17.93±3.26	0.34±0.05	13.42±1.86
FN38	0.38±0.23	46.49±3.75	0.63±0.23	21.09±1.54
VCAM-1	0.24±0.13	139.17±7.87	0.30±0.05	75.11±13.97
B. SF				
Medium	0.42±0.34	34.84±1.89	1.33±0.66	50.74±3.45
FN38	0.54±0.18	71.59±2.55	1.39±0.86	86.53±7.30
VCAM-1	0.32±2.33	97.62±2.07	2.11±0.76	164.10±13.48

Proliferative response of PB and SF T cells from RA patients to immobilized CD3 mAb combined with either FN38 or rsVCAM-1. Plates were coated with the different proteins as described under Methods section. Results (in cpm $\times 10^{-3}$) represent the medium average of triplicate values plus standard deviation.

ied were receiving different therapies, the pattern of adhesive reactivity observed was very similar in all of them, which strongly suggests that the observed phenomena were related to disease itself, and not their treatment, age, duration, and evolution of the disease. This fact is also extended to the four patients receiving corticosteroid therapy.

The accumulation of T lymphocytes in the RA SM plays an important role in the development of inflammatory events that leads to articular damage and destruction (1–4). In the early steps of RA, a characteristic feature is the neovascularization of the SM and the formation of perivascular lymphocyte infiltrates (1–4). Additionally, the lining layer, which separates SM from the articular cavity and the SF, undergoes a significant hyperplasia which probably bears relationship to T cell infiltration (1–4). Nevertheless, the mechanisms responsible for the development of these lesions are largely unknown.

The emigration of lymphocytes from PB to SM requires their prior binding to EC. It is known that a number of ligands (ICAM-1, VCAM-1, ELAM-1, CD62/GMP140/PADGEM, etc.) are induced on EC surface in response to inflammatory agents (reviewed in references 5 and 6). Recent reports indicate that initial interactions of neutrophils with EC involve the CD62- and ELAM-1-mediated rolling of cells on the vessel wall (12, 14). The integrins seem to participate in the next step of cell traffic, which is a reinforced adhesion, the cellular extravasation and migration into inflamed tissue which occur subsequent to leukocyte-EC selectin-mediated contacts (12, 14). All these sequential processes have been reported for neutrophils, although the interaction of T cells with either in vitro-activated or chronically inflamed EC in skin using an ELAM-1-mediated mechanism has also been reported (21–23, 49).

Our results show that synovial T cells display a higher adhesiveness to ELAM-1, as compared with PB T cells. Most of such synovial T cells express a memory CD45RO+ phenotype (33, 35, 37), a feature that has been correlated with the T cell binding to ELAM-1 (22). The level of ELAM-1 expression by EC from inflamed synovium is reported to be similar to that found in other chronic inflammatory processes, although lower than that reported from chronic skin lesions (49). In that latter setting, a specific T cell subset displaying ELAM-1 binding ability has been described to bear the CLA-HECA 452 antigen (49). Whether this synovial T cell subpopulation binding ELAM-1 could also express that marker, or other synovium-restricted antigens, requires further studies. It is noteworthy that the HECA 452 antigen has been detected on dendritic cells and EC of SM from RA (60).

In a second step of cellular migration, leukocyte interaction with EC involves the integrin family members. These adhesion molecules would allow for a reinforced cell attachment and then the transendothelial migration (11, 12, 14). According to current knowledge, integrin-mediated T cell binding to activated endothelium involves mostly LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions (7). The expression of ICAM-1 in RA synovial tissues is known to be characteristic not only for EC but also macrophages, lining layer cells, and some lymphocytes (50, 61). Studies on the role of LFA-1/ICAM-1 interactions in synovial lesions of RA patients are currently underway in this laboratory. Interestingly, ICAM-1 has been found to play a role in the pathogenesis of other chronic autoimmune disease such as murine lupus nephritis (62).

Our results clearly demonstrate an increased VLA-4-mediated binding to VCAM-1 by most synovial T cells. This enhanced adhesion to VCAM-1 could be related to their activated (AIM/CD69+, DR+) and memory (CD45RO+) phenotype (32–37), since it has been recently reported that this interaction is enhanced upon in vitro activation of T cells, particularly those possessing a memory phenotype (52).

Finally, our data suggest the existence of several levels of cellular affinity for VLA-4 ligands. Thus, the enhanced binding of synovial T cells to both VCAM-1 and FN38 reflects an in vivo upregulated state of affinity. However, a further increase in SF T cell binding to both VLA-4 ligands could be achieved using additional in vitro stimuli like phorbol esters, or the engagement of the CD3-TcR complex. Thus, those stimuli can further enhance the ligand binding of cells that have already developed binding activities in vivo (37). In addition, to bind VCAM-1, VLA-4 mediates homotypic cell contacts in a VCAM-1-independent manner (63, 64). Whether this adhesive property of VLA-4 is also upregulated in synovial T cells is currently under investigation.

Both ELAM-1 and VCAM-1 might participate in subsequent steps of T cell adhesion to EC and migration into the SM of RA. Very recently, it has been described that neutrophil interaction with ELAM-1 activates Mac-1, thus enhancing the avidity of this integrin to bind its ligands (14). It is known that memory and naive T cells exhibit different recirculation pathways, with memory CD45RO+ T cells leaving the bloodstream through the peripheral tissue endothelium (65). It could be hypothesized that a subset of CD45RO+ PB T cells in RA patients interacts with ELAM-1 on activated EC in inflamed tissue. This interaction could then increase VLA-4 (and possibly LFA-1) avidity for VCAM-1 (possibly for ICAM-1), allowing T cells to enter into inflamed synovium. The adhesion and transmigration of lymphocytes through EC is known to be regulated by cytokines, inflammatory mediators, and chemotactic agents (5, 6, 66, 67). It was recently suggested that ELAM-1 could play a chemoattractant role directing the movement of leukocytes (14). The pathological significance of an enhanced binding of SM and SF T cells to ELAM-1, that is restricted in RA synovial tissue to the EC, after their entry into SM, remains to be determined.

VLA-4 also participates in T cell binding to FN, and this interaction is enhanced upon in vitro cellular activation (29, 30, 53, 54). Accordingly, we have previously documented an increased binding of synovial T cells to FN in RA patients (37). SM and SF from RA have been reported to contain high concentrations of FN (68). Therefore, T lymphocyte interaction with FN in the SM could constitute an important feature in the infiltration and maintenance of cells perpetuating the tissue injury in this compartment (37, 69), and can convey proliferative signals to infiltrating T lymphocytes (see above and 55–58). The high expression of VCAM-1 by the RA lining layer could also be involved in the migration of infiltrating cells to the lining, thus participating in its hyperplasia and in the resulting increased cellularity of SF. We have found that the interaction between VLA-4 and VCAM-1 also delivers proliferative signals to synovial T cells. This signaling pathway, probably acting together with that of LFA-1/ICAM-1 reported in normal T cells (70), could represent important mechanisms in synovial T cell proliferation. The interaction of T lymphocytes with the synovial antigen-presenting macrophages, expressing high levels of VCAM-1 (51), is thought to participate in the initiation of the immune response in the RA synovitis (2, 71).

The pathogenesis of tissue injury in RA remains obscure.

We believe that the results presented here shed new light on the immune mechanisms responsible for tissue infiltration in this disease, highlighting the possible role of adhesion molecules in directing T cell traffic to synovial tissue, and enhancing their in situ localization, activation, and proliferation.

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CAPITULO IV

MOLECULAS DE ADHESION IMPLICADAS EN LAS INTERACCIONES DE CELULAS T CON SINOVIOCITOS TIPO FIBROBLASTO EN ARTRITIS REUMATOIDE: DIFERENTES MECANISMOS DE REGULACION POR CITOQUINAS EN AMBOS TIPOS CELULARES

Los diferentes tipos celulares dentro del microambiente sinovial están sujetos a múltiples interacciones en las que las citoquinas desempeñan un papel regulador esencial. Estudios de microscopía electrónica han identificado la existencia de contactos entre linfocitos T y células fibroblásticas en la membrana sinovial de AR.

Con el fin de conocer estas interacciones hemos diseñado ensayos de adhesión *in vitro* de células T, obtenidas de diferentes compartimentos de pacientes con AR, a sinoviocitos tipo fibroblasto (STF) en cultivo, también de AR. Se ha investigado la implicación de varios pares ligando-receptor y la regulación de estas interacciones por la acción de diferentes citoquinas sobre células T de SP y sobre STF. Aproximadamente un 10% de células T de SP tanto de pacientes como de controles sanos se unen a STF en condiciones basales, mientras que un número al menos 3 veces mayor de células T en LS y MS presentan esta capacidad (Fig. 1). La preincubación de STF durante 24 h con IFNγ, TNFα, IL-4 o IL-1β, incrementó significativamente la adhesión de linfocitos T de SP por orden decreciente de potencia (Tabla 1). La estimulación prolongada de células T de SP con las mismas citoquinas también resultó en un aumento de la adherencia que fué significativo para IFNγ, TNFα e IL-4 (Fig.2). Los estudios de inhibición con AcM frente a moléculas linfocitarias demostraron que VLA-4, LFA-1 y en menor medida CD2 están implicadas en estas interacciones, en linfocitos T de los 3 compartimentos (Fig. 3). Los AcM frente a VCAM-1, ICAM-1 y LFA-3 en STF también inhibieron significativamente la adhesión en los 3 compartimentos (Fig. 4). La vía de adhesión VLA-4/VCAM-1 parece tener mayor preponderancia en la adhesión aumentada de linfocitos T de LS y MS aunque las diferencias respecto a su participación en SP no llegaron a alcanzar significación estadística (Fig 3 y 4). Se detectó un aumento de expresión de VLA-4, LFA-1 y CD2 en las células T del LS respecto a las de SP (fig 5) probablemente en relación con el fenotipo de células T de memoria mayoritario en el

compartimento sinovial. Sin embargo, este aumento de expresión, en términos de intensidad de fluorescencia relativa, no fué suficiente para explicar los incrementos en la adhesión, lo que sugiere cambios conformacionales en los receptores de las células T sinoviales que en su mayoría presentan fenotipo activado (Ver capítulos I, II y III) . A diferencia de la estrecha correlación que existe entre el aumento de moléculas inducido por citoquinas en STF y su capacidad de adhesión, la adhesion inducida por citoquinas en linfocitos T de SP no se correlacionó con los cambios en la expresión de las moléculas de adhesión implicadas (fig. 6), por lo que las citoquinas podrían estar mediando cambios cualitativos en estos receptores.

Estos resultados indican que las 3 vías de adhesión molecular estudiadas, LFA-1/ICAM-1, VLA-4/VCAM-1 y CD2/LFA-3 están implicadas en las interacciones de células T de AR a STF. Las citoquinas podrían estar regulando estas interacciones por mecanismos diferentes en ambos tipos celulares.

"Molecular adhesion pathways involved in rheumatoid T cell-fibroblast like synoviocyte interactions: different cytokine regulatory mechanisms on both cell types"

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ABSTRACT

T lymphocytes accumulate in the Rheumatoid Arthritis (RA) synovium where they interact with other cell types. We have developed *in vitro* binding assays to study the adhesion between T cells from various sources and cultured RA fibroblast-like synoviocytes (FLS). The role of several receptor ligand-pairs and the influence of cytokines on both cell types was analized. About 10% peripheral blood (PB) T lymphocytes from either RA patients or healthy donors bound to RA FLS, whereas three folds greater number of cells in the synovial fluid (SF) or synovial membrane (SM) showed this capacity. Preincubation of FLS or PB T cells with IFNγ, TNFα, IL-4 or IL-1β (ranked in decreased order of potency) resulted in enhanced adherence between these two cell types. Preincubation of T lymphocytes with monoclonal antibodies (Mab) showed that VLA-4, LFA-1 and to a lesser extent CD2 were involved in T cell-FLS adhesion. Comparable results were obtained when Mab against the ligands of these molecules on STF (VCAM-1, ICAM-1 and LFA-3) were used. Quantitative changes in the receptors on the synovial T cells did not reach highly enough levels to explain the enhanced adhesion to FLS. Despite of the close correlation between adhesion and surface molecule upregulation on cytokine stimulated FLS, enhanced adhesion induced by cytokine preincubation of PB T lymphocytes did not parallel surface molecule expression. These data suggest that a qualitative change in T cell molecules could be partially responsible for this effect. We conclude that VLA-4/VCAM-1, LFA-1/ICAM-1 and to a lesser extent CD2/LFA-3 are implicated in RA T cell-FLS interactions. The differences in adhesion capacity between PB and synovial compartments T cells can be related to the predominant activated and memory population in synovial T cells with quantitative as well as qualitative changes in adhesion molecules. Cytokine-mediated regulation of these interactions on PB T cells and FLS is mediated through different mechanisms.

INTRODUCTION

T lymphocyte infiltration is one of the hallmarks of Rheumatoid synovitis. Accumulation of these cells, derived from the bloodstream, appears to be the result of multiple adhesive events with a complex regulation. It is conceivable that, after prior adhesion to endothelial cells, T cells enter in the synovial membrane (SM) where they interact with extracellular matrix (ECM) proteins or with other cell types.

A large basic knowledge about the role of adhesion molecules in T cell interactions has been pyled up during the last years (1-3) and their role in inflammatory processes has been stablished (4-6). To date, most functional studies about adhesion receptors in RA have centered in T cell-EC interactions (7,8) or attachment of T lymphocytes to ECM protein FN (9,10). Besides electron microscopy observations of synovial fibroblast-T cell contacts into SM (11) some *in vitro* evidences of mutually stimulatory interactions of T cells and fibroblasts have been demonstrated (12). Holoshitz et al. have described an *in vitro* system in which single cell suspension of lymphocyte and synovial cells from joints of RA patients were cultured in presence of mycobacterial antigen and IL-2, and produced an outgrowth of an organized inflammatory tissue with an ECM and histologic features of pannus (12). The presence of select lymphocyte adhesion molecules and their possible ligands in synovial lining cells has been well defined in synovial tissue (9,13-15). However, receptors that mediate these interactions *in vivo* and the factors affecting their regulation are only partially knwoun.

Earlier studies with monoclonal antibodies (MAb) had identified LFA-3 as the first receptor on cultured fibroblast like synoviocytes (FLS) involved in the interaction with peripheral blood (PB) T lymphocytes through its ligand CD2 (16). Subsequent investigations have failed in demonstrating LFA-3 mediated adhesion, but supported the participation of ICAM-1 (CD 54) (17), a molecule largely expressed by inflammed tissue linning cells in RA (13,14). Upregulated expression of ICAM-1 on RA FLS has been described after stimulation with cytokines like IFNγ, TNFα, IL-1ß or IL-4 (17-19). By contrast, they had no effect on LFA-3 expression (20). The leukocyte integrin LFA-1 (CD11a/CD18) is the counterreceptor for ICAM-1 in lymphocytes (21). However, adhesion was

only partially inhibited by anti-ICAM-1, anti-CD11a or anti-CD18 MAbs, suggesting the existence of ICAM-1 independent adhesion mechanisms.

Interestingly, VCAM-1, a cytokine inducible endothelial cell molecule (22), has been detected in the synovial lining of RA (14,15). Constitutive expression of VCAM-1 in cultured FLS is further increased by exposure to IL-1ß, IL-4, TNFα and INFγ (18). VCAM-1 interacts with the lymphocyte integrin VLA-4 (21,23) an integrin upregulated in memory T cells (24). We have previously demonstrated that SF and SM T cells from RA patients bind more efficiently to purified VCAM-1 than do PB T cells (7). Relevance of this adhesion pathway is reinforced by the capability of VCAM-1 to trigger costimulatory signals in T lymphocytes through VLA-4 (25). Recently, it has been reported that the increased expression of VCAM-1 on IL-4 stimulated synovial cells considerably enhances adhesion of PB T cells (26). However, to our known, cytokine-mediated regulation on T cells has not been assesed.

The aim of the present study has been to assess the role of various surface adhesion molecules in the interactions between PB, SF and SM T lymphocytes and FLS from RA patients. Cytokine regulated adhesion have been settled by separate analysis of their effects on PB T lymphocytes or FLS.

PATIENTS AND METHODS

Patients. Eight female and one male patients (mean age 59 years, range 33-72) were investigated. All of them fulfilled criteria for the diagnosis of RA according to the American College of Rheumatology (27). Seven patients were taken non steroidal antiinflammatory drugs and two patients were receiving low doses of oral prednisone. In addition five patients were taken methotrexate and two were treated with gold compounds. After consent, SF and PB were collected from eight of them and synovial tissue for T cell isolation were obtained at the time of joint surgery from four patients.

Synoviocyte culture. Synovial membranes obtained at the time of surgery from RA patients were minced and treated for 2 h at 37° with 2mg/ml of type P collagenase (Boehringer Manheim Diagnostics, GMBH, Germany) in serum free RPMI 1640 culture medium (Flow Laboratories, Inc, Irvine, Scotland). Cells were then filtered through a nylon mesh, extensively washed and cultured in DMEM (Gibco, Grand Island, NY) suplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50 U/ml penicillin and 50 mgr/ml streptomicin (all from Flow Laboratories Inc., Irvine, Scotland) in a humidified 5% C02 atmosphere. After overnight incubation, non adherent cells were removed and adherent cells were cultivated in DMEM plus 10% FCS. At confluence, cells were trypsinized, split at a 1:3 ratio, and recultured in medium. Synoviocytes were used from passages 3 through 9 in these experiments, during which time they were a homogeneous population of fibroblasts-like synoviocytes (<1% CD11b+, <1% Fc-gamma RII receptor +).

Preparation of purified T lymphocytes. PB and SF samples were collected at the same time from heparinized tubes. T cells (>90% CD3+ and <2% CD11b+ and CD19+) were purified from mononuclear cells obtained after Lymphoprep (Nycomed Pharma AS, Oslo, Norway) gradient centrifugation and removal of adherent cells on plastic flasks (Costar Corp., Cambridge, MA). Purification was achieved by passage though nylon wool columns (Dupont/NEM, Boston, MA).

PB T cells from sixteen healthy donors were obtained as described for RA patients T cells.

Reagents and monoclonal antibodies. Phorbol myristate acetate (PMA) was purchased from Sigma (Sigma Chemical Co, St. Luis, MO). Recombinant human (h) IL-1β (specific activity $5x10^8$ U/mg, purity>95%) was purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant hTNFα(spec.act. $5x10^7$ U/mg, purity>95%)was provided by Genentech (South San Francisco, CA). Recombinant hIFNγ (spec.act. $2x10^7$ U/mg, purity>95%) was a gift from Amgen Biological. Recombinant hIL-4 (spec.act. $2x10^7$ U/mg) was purchased from Promega (Madison, WI). In each case, the final concentration of LPS in culture after dilution of recombinant cytokine was<0.005ng/ml.

The following MAb were used and have been previously described: anti-CD3 SPV-T3b, anti-CD45 D3/9, anti-CD11b Bear-1, anti CD11a TP1/40, anti-CD18 TS1/18 , anti α4 (CD49d) HP2/1, TS2/16 and Lia 1.2.1 both directed againts β1 subunit (CD29), and P3X63 myeloma culture supernatant as a negative control, (7,9); OKT11 anti-CD2 (28), TS2/9 anti-LFA-3 (29), RR1.1 anti-ICAM-1 (CD54) (30), W6/32 anti HLA A-B (20). Anti-VCAM-1, 4B9, was the generous gift of Dr. Harlam (University of Washington, Seattle, WA).

Flow cytometry analysis. Fluorescence flow cytometry analysis was performed on a FACscan cytofluorometer (Beckton & Dikinson, Mountain View, CA). Resting and stimulated cells were incubated at 4°C with hybridoma cultured supernatants, following by washing and labeling with FITC-conjugated goat anti-mouse Ig (Dakopatts). Data were collected in linear and sometimes in logaritmic scales. Five thousand of cells were analyzed for each sample. In every experiment, the

sample with the great fluorescent intensity was analized first, and the fluorescent gain was adjusted so that 1-5% of cells were positive in the highest fluorescent channel. A negative control was then analized, and the upper limit of negativity was stablished by determining the lowest channel that include 95-99% of negative control cells. Because the fluorescence conditions were diferent from experiment to experiment, data were normalized to express mean relative fluorescence (rMFI) as follows: rMFI=absolute $MFI_{cytokine}$/absolute MFI_{medium} after substracting from each MFI the MFI of the negative control (P3X63).

Cytokine treatment of cells and cell attachment assays. Culture synoviocytes from passages 3 throuhg 9 were cultured in 96-well flat-bottomed microtiter plates (Linbro, Flow) ($15x10^3$ synoviocytes/well) for 24-48h in DMEM-10% FCS. When indicated IL1-β (100U/ml), TNFα (500ng/ml), IL-4 (100U/ml) or IFNγ (100U/ml) were added for 48h. Thereafter, plates with confluent monolayers of FLS were washed with RPMI 1640 and resting or stimulated T cells ($3x10^6$/well) were added in serum free medium and incubated for 1 hour at 37°. Stimulated T cells were cultured for 24h-7days in RPMI supplemented with 10% FCS, 2mM L-Glutamine and antibiotics in presence of each cytokine or medium alone at doses indicated for FLS. After incubation for allowing T cells to adhere to FLS, plates were washed three times and examined in an inverted microscope. Each condition was performed in triplicate. Within each well cells for at least three diferent fields were counted. The number of cells counted in a non washed well was considereded as theoretical maximum binding. In inhibition assays, cells were preincubated for 30 min at 4° (T cells) or at room temperature (FLS) with the specific MAbs or the control X63 MAb.

Statistical analysis. Statistical analysis was performed using Student's test for paired samples in the StatWork-TM data base program

RESULTS

Adhesion of T lymphocytes from different sources to unstimulated FLS.

Initial experiments were designed to measure baseline adhesion of T lymphocytes isolated from different compartments of RA patients and PB of controls.

As shown in figure 1, a similar proportion of PB T cells from healthy donnors (x±SE=10±1%) and RA patients (8.5±1%) bound to unstimulated FLS *in vitro*, without significant differences in adhesion. When T cells were isolated from synovial compartments we found that 31±3% SF T cells and 26±2% SM T cells were able to bind to cultured FLS. Those data support and increase in adhesion at least three folds greater than that exhibit by PB T cells in the same patients (Fig.1).

Effect of individual cytokines on FLS-T lymphocyte adhesion.

In an attemp to explain the increased adhesion of synovial T lymphocytes to FLS we independently explored the effect of some cytokines either on PB T cells or FLS. Pretreatment of cultured RA FLS with IL-4 (100 U/ml), TNFα (500 ng/ml) and IFNγ (100 U/ml) resulted in enhanced adherence of PB T lymphocytes ranked in increasing order of potency. IL-1ß (100 U/ml) had a variable effect with moderate or no increments in the ability of FLS to bind T cells (See median data from various experiments in table 1). No statistical differences were found in the attachment of PB T cells from controls or RA patients. Cytokine concentrations and time of incubation shown were selected after preeliminary experiments of dose-response and time-course of these effects (Data not shown). Stimulation with PMA was used as a positive control of adhesion.

Because IFNγ was the most potent enhancer of binding for PB T cells we studied attachment of SM T cells to FLS stimulated with this cytokine. We just found 20% increment in adhesion over untreated (medium) FLS (data not shown).

To investigate the effect of cytokines on T lymphocytes and its potential consequences in adhesion we treated PB T cells with the same doses of cytokines described above. Figure 2 shows data from various experiments in which T cells were exposed to cytokines for 24 h and 7 days. After this prolongued stimulation, binding was significantly rised by IL-4, TNFα and IFNγ, although increments did not reach levels induced by FLS stimulation. Time of incubation was choosed after time-course experiments to obtain optimal stimulatory effects (data not shown).

Effects of MAb on PB T lymphocyte-FLS adhesion.

To asses the role of several adhesion molecules in T lymphocyte-FLS interactions we tested the effect of MAbs directed against ligands on both cell types.

Data in figure 3 shows the mean % adhesion when T cells were preincubated with MAb and added to unstimulated FLS. The antibodies directed against α or ß chains of the same heterodimer, anti-LFA-1α/anti-ß2 or anti-α4/anti-ß1, were equally effective in reducing adhesion without any additive effect. When MAb were used separately, anti-α4 and anti-ß1 reached the highest

reduction in the number of attached cells ($p \leq 0,01$), followed by anti LFA-1a and anti-ß2 ($p \leq 0,001$) and a modest but significant effect of anti-CD2 MAb ($p \leq 0,05$). The optimal inhibitory effect was achieved by a combination of anti-α4 and anti-LFA-1α MAbs which were able to block 74±2 % of binding. Figure 3 shows as baseline condition the effect of an irrelevant isotype matched antibody X63. The addition of this MAb had no interference on PB T lymphocyte adhesion when compared with cells in absence of any antibody, so subsequent adhesion assays will be referred to X63 as control condition unless otherwise is indicated. As an additional control for antibody specificity we tested the effect of D3/9 (CD45) MAb in T cells, and no changes in adhesion were detected.

No signicant differences in MAb inhibition were observed when PB T cells were isolated from RA patients or controls .

To determine the contribution of FLS ligands in these interactions, we performed paralel assays preincubating FLS with anti-ICAM-1, anti-VCAM-1 and anti-LFA-3 MAb. Significant reduction in the attachment was observed with the three MAb (Fig. 4). The higher blockade in adhesion was obtained in the presence of anti-ICAM-1 ($p \leq 0,001$) and anti-VCAM-1 MAb ($p \leq 0,01$) which correlates with data obtained with anti-α4 and anti-LFA-1α MAb on T cells. Inhibitory effect of anti-LFA-3 MAb ($p \leq 0,01$) was quite similar to that obtained with anti-CD2 in PB T cells. The specificity of these inhibitory effects was accomplised by treatment of FLS with anti-HLA A-B MAb (W2/36), which had no effect in T cell binding. (Data not shown).

Effects of MAb on SF and SM T lymphocyte-FLS adhesion.

Basal adhesion of SF and SM T lymphocyte to unstimulated FLS was increased about 3-4 folds over that showed by correlative PB T cells (Fig. 1). So, we developed the same protocols to asses the contribution of each adhesion pathway in these enhanced adhesion. The percentages of adhesion obtained with the MAbs directed against LFA-1 α and β chains, and CD2 were quite similar than those reached for PB T cells ($p \leq 0,01$ and $p \leq 0,05$ respectively) as is illustrated in figure 3. Anti-ICAM-I and anti LFA-3 MAb also abrogated a significant proportion of SF ($p \leq 0,01$ and $p \leq 0,05$ respectively) and SM ($p \leq 0,05$ for both MAb) T cell adhesion (fig 4). It is noticeable the capabilility of both anti-α4 and anti-VCAM-1 MAbs to inhibit adhesion of a higher proportion of T cells in SM and SF (see figures 3 and 4) when compared to PB inhibitory effects, although these differences do not reach statistical significance ($p=0,2$)

Adhesion molecule expression by T lymphocytes and effect of cytokines.

Immunofluorescence flow cytometry analysis of molecule expresion were carried out in T lymphocytes from PB and SF from 8 RA patients. No differences were found in the number of positive cells for any of the molecules studied (CD11a, β2, α4, β1 and CD2), and all of them were expressed in 95-100% T cell population of both compartments. The increments in the MFI detected in SF T cells (rMFI between 1,2-2,1 in all molecules studied related to PB MFI) were not enough to explain completely the three fold enhanced adhesion (see Fig 1). Fig 5 shows characteristc flow cytometry histogram profiles of these receptors on PB and SF T cells from a representative patient.

Upregulatory effect of IL-1ß, IL-4, TNFα and IFNγ is well known on ICAM-1 and VCAM-1 expression by human FLS (17-19). Herein, we have studied the influence of these soluble mediators on LFA-1, VLA-4 and CD2 expression by PB T cells. Figure 6 shows variations in fluorescence intensity (mean±SE rMFI) for each molecule studied. Cytokines were used at the same doses described for adhesion assays. No meaningful changes were observed when cells were stimulated for 24 h; although moderate MFI increments can be observed in some of the experiments during prolongued stimulation, none of these variations resulted in significant upregulation.

DISCUSSION

There is increasing *in vivo* evidence for the key role of adhesion molecules in inflammatory processes such as RA. The interactions between synovial lining cells and T lymphocytes, both overrepresented in the pathologic SM of RA, are likely to be a fundamental aspect in the development of inflammed tissue which leads to destruction of the joint.

Our data show that the three molecular recognition systems studied are involved in these adhesion events: LFA-1/ICAM-1 and VLA-4/VCAM-1 have resulted to be particularly prominent but there is undoubtely a role for CD2/LFA-3. Because of poor adhesion of resting PB T cells to synovial fibroblasts, there must be several mechanisms to explain the dramatic augment in the number of interacting cells we have found in SF or SM.

Some explanations can be inferred from the regulated expression of FLS molecules .Cuantitative changes in the expression of ICAM-1 and VCAM-1 molecules between normal and pathologic fibroblasts has been reported. Increased expression of ICAM-1 have been found in scleroderma (31) and other sites of cutaneous inflammation (32), and the amount of

VCAM-1 and ICAM-1 expression in the synovial lining of RA is significantly higher than those in normal synovium (14). This upregulated expression is expected due to the presence of high concentration of inflammatory cytokines in the synovium. Constitutive VCAM-1 expression on cultured FLS was increased by IL-1β, TNFα, IL-4 and IFNγ (18,19). These cytokines with the exception of IL-4 also increment ICAM-1 expression (18). LFA-3 expression is not affected by cytokines but the constitutive concentration of this antigen on FLS is higher than those displayed for VCAM-1 or ICAM-1 (20). The production of IL-1β and TNFα within the RA joint have been confirmed histologically using *in situ* hybridization technique and inmunostaining (33, 34). Quantitative analysis of cytokine gene expression have also been performed (35). Monocyte/macrophage lineage cells which appear to be the main source of these cytokines localize throughout the intertitial space and within the synovial lining layer (33). In contrast, other cytokines produced by T cells, such as IFNγ or IL-4 are virtually indetectable by these procedures, despite the presence of high number of infiltrating T cells (36, 37). Interestingly, a recent study, based in a new inmunohistochemical intracellular staining of cytokines, have detected a substantial presence of T cell derived cytokines (IL-2, IL-4, IL-5, IL-10, IL-13, IFNγ and TNFβ) at a protein level in synovial biopsy samples obtained from active inflammation in RA patients (40). These data indicates that more T cell derived cytokines than previously recognised can be present in active synovitis. In addition, it is conceivably that neither IL-4 nor IFNγ would likely be present in inflammatory tissues without the simultaneous presence of other cytokines and cytokine combinations might have important modulative effects. In the better studied system of cultured EC, IFNγ+TNFα and IL-4+TNFα were better than TNFα alone in stimulating EC adhesivennes for T cells but not for neutrophils (39). IL-4 increased the capacity of TNFα to induce endothelial VCAM-1 expression and the additive effect of the two cytokines on EC adhessiveness for T cells was mainly mediated through VCAM-1 (39).

Previous studies have described cytokine-mediated upregulation of FLS surface molecule expression that correlates with the levels of augmented T cell adhesiveness induced in the FLS by the same cytokines. However in our *in vitro* system, IL-1β had a variable effect, with lower increments above basal adhesion than expected from the expression levels of FLS molecules induced by the cytokine (17-19) Discrepances between the expression and function of ICAM-1 induced by IL-1ß have been communicated previously in dermal fibroblasts (40, 41), suggesting the existence of additional levels of regulation as well as quantitative alteration of ICAM-1 molecule.

Although cytokines have been demostrated to play a definite role in the expresion of adhesion ligands on endothelial and other non lymphoid cells, less is known on their influence in T cells. We have demonstrated that IL-4, TNFα and IFNγ treated PB T cells exhibit an enhanced adhesiveness to unstimulated FLS than resting PB T cells. However, changes in the level expression of molecules on T cells did not paralel changes induced in adhesion by the same cytokines. These findings strongly suggest that qualitative as well as quantitative changes might be induced in T cell counterreceptors. Closely related with this issue, we have reported recently the induction of an activation-dependent epitope of the β1 integrin on PB T cells after prolongued stimulation with TNFα and IFNγ (42) This enhanced neoepitope expresion correlated with a higher binding ability to fibronectin of cytokine-activated T cells (42). Moreover, the presence of this activated conformation of β1 integrin could be detected in SF and SM T cells of RA patients (42). So, our *in vitro* data in the present study suggest that, at least VLA-4-T cell mediated interactions, might be regulated *in vivo*.by cytokines.

Some additional explanations can be considered to discuss functional differences among T lymphocytes from PB and synovial compartments in their adhesion to unstimulated FLS. Data from inmunofluorescence flow cytometry analysis indicated that no significant differences in the percentage of positive cells were found in T cells from different sources when stained with anti-LFA-1α, anti-ß2, anti-VLA-4α, anti-ß1 or anti-CD2 MAbs. However upregulated expression of these molecules in terms of MFI were detected in synovial T cells. Since these molecules have been shown to be expressed at enhanced levels on memory T cells (24,43), it is not surprising that this characteristic adhesion phenotype was the dominant in synovial tissue where memory T cells predominate (7,9,10,44). Some interesting functional consequences of this phenotype have been communicated previously. Memory T cells have an enhanced capacity to adhere to ECM proteins by upregulated expression of their VLA ECM receptors (24). The intrinsic migratory capacity of memory T cells (CD45RO+, CD45RA-, $CD29^{high}$, $CD11a^{high}$) have been recently reported in an *in vitro* model of transendothelial migration. Therefore, a subset of CD45RA- T cells in PB of RA patients manifiests a greater capacity for migration than control PB T cells, which even is enhanced in T cells recovered from the synovium (45). In related studies, the percentage of naive CD45RA+ cells in the PB T cell population which adhere to IL-1β and IFNγ stimulated FLS was lower than that on the nonadherent population (46).

In addition to memory T cell predominance, most of T lymphocytes in sinovial compartments are activated (7,9,10). Interestingly, high affinity binding of integrin molecules to their ligands have been described after activation of T cells; integrin molecules expressed by resting T cells are unable to bind to their ligands, requiring stimulation of the T cell to adquire functional

competence (revised in 21, 47). In this context, we have demostrated previously the upregulated function of VL5 FN receptors on SF T cells from RA patients (9). Thus, most T cells in a resting state in PB do not bind to FLS albeit almost 100% population expresses the integrins LFA-1 and VLA-4. By contrast, an expanded pool of memory and activated T lymphocytes, bearing active receptors, may contribute to the enhanced adhesion found in the synovial compartments. In fact, activation could explain the increments in T cell attachment to FLS that cannot be supported only by the increments found in synovial T cell molecule expression.. Accordingly, RA SF and SM T cells exhibited an enhanced adhesiveness to FN and VCAM-1 than did PB T cells, by an upregulated expresion and function of VLA-4 integrin (7,10).

Finally, relevance of adhesion molecules is supported not only in their adhesive properties, but in their capability to act as signal transducers. CD2, LFA-1 and VLA-4, all have transmembrane signalling properties which may converge to produce several cell responses (Reviewed in 48-50). LFA-3 molecule can synergize with a second endogenous CD2 ligand to promote physiologic antigen-independent T cell triggering (48). Inmovilized MAb to LFA-1 or VLA-4 synergize with anti-CD3 antibodies to induce T cell proliferation, as do their respective ligands ICAM-1 and VCAM-1 (25,51). No less important consequences of this signalling could be the induction of specific gene expression by ligand or antibody binding to adhesion molecules. LFA-3 on monocyte and thymic epithelial cells has been suggested to be involved in mediating IL-1 release and production (52). Subsequently, T cell adhesion to accesory molecules via CD2/LFA-3 might result in augmented accesory cell cytokine production. The induction of collagenase and stromelysin gene expression in synovial fibroblast has been described by signalling through the VLA-5 integrin (53). Futhermore, β1 integrin-mediated interaction with ECM proteins has been demonstrated to have an important role in regulating persistent cytokine gene expression in RA SF mononuclear cells (54). Those data indeed emphasize the regulatory function of T cells in cytokine production by other cells.

Cellular interactions mediated by the ligand pairs studied herein might play very important roles in the effector function of both cell types involved. The proinflammatory cytokines in which rheumatoid tissue is enriched can modulate the expression and function of these molecules by different mechanisms.

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Table 1. Adhesion of PB T lymphocytes to cytokine stimulated FLS from RA patients

CYTOKINE	% Δ Adhesión (mean±SEM)	n
Medium	0	4
IL-1β (100U/ml)	19 ± 20	4
IL-4 (100U/ml)	97 ± 24 *	4
TNFα (500ng/ml)	122 ± 2 **	4
IFN γ (100U/ml)	176 ± 40 *	8
PMA (20ng/ml)	300 ± 28 *	4

FLS were preincubated with cytokines and PMA for 24h. Results are expressed in percentage of increased adhesion over the effect of medium. The % Δ adhesion was calculated as follows: $[(Adhesion_{citokine}/Adhesion_{medium})-1] \times 100$. Statistical significance: * $p \leq 0,01$; ** $p \leq 0,001$.

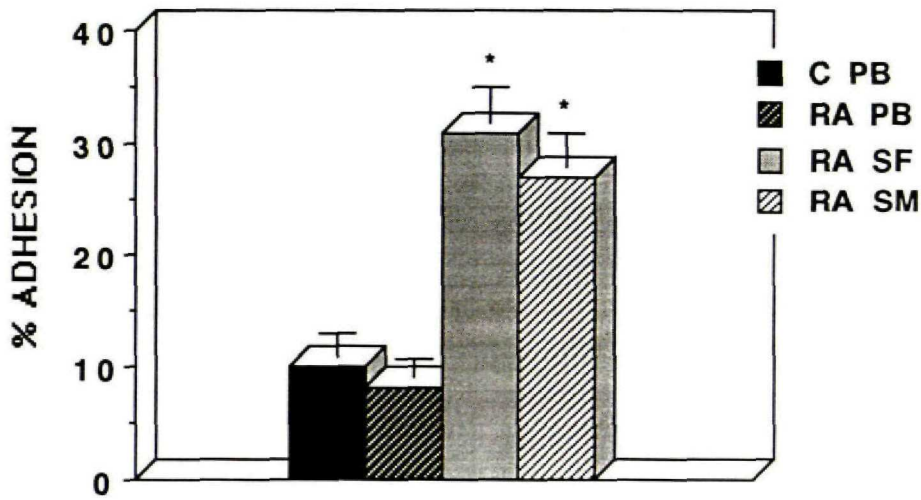

Fig.1. Baseline adhesion of T lymphocytes from different sources to unstimulated RA FLS. Freshly isolated T cells from RA SF (n=7) and RA SM (n=4) showed an increased binding capacity compared to PB T cells from controls (n=11) or RA patients (n=7). Results are expressed as the mean±SEM from separate experiments. Statistical significance: * $p < 0{,}001$.

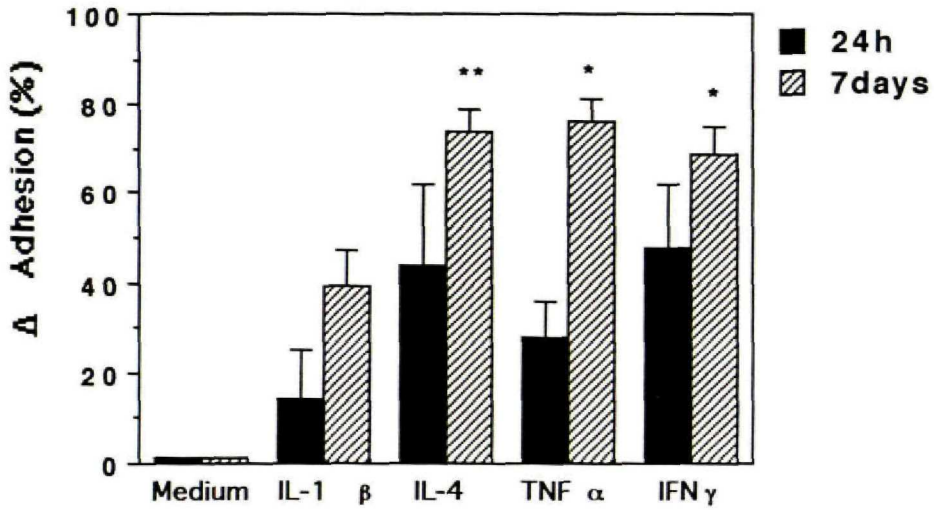

Figure 2. Effect of cytokine treatment of PB T lymphocytes in their adhesion to unstimulated FLS. T cells were cultured with each cytokine or medium alone for 24 hours (solid bars) or 7 days (striped bars). Doses of cytokines were the same used for FLS stimulation in other experiments and are described under Material and methods. Results are expressed in percent of increased adhesion over the effect of medium. Values represent mean±SEM from 4 separate experiments. Only prolongued stimulation for 7 days resulted in statistically significant increments of adhesion: *$p \leq 0,05$; **$p \leq 0,01$.

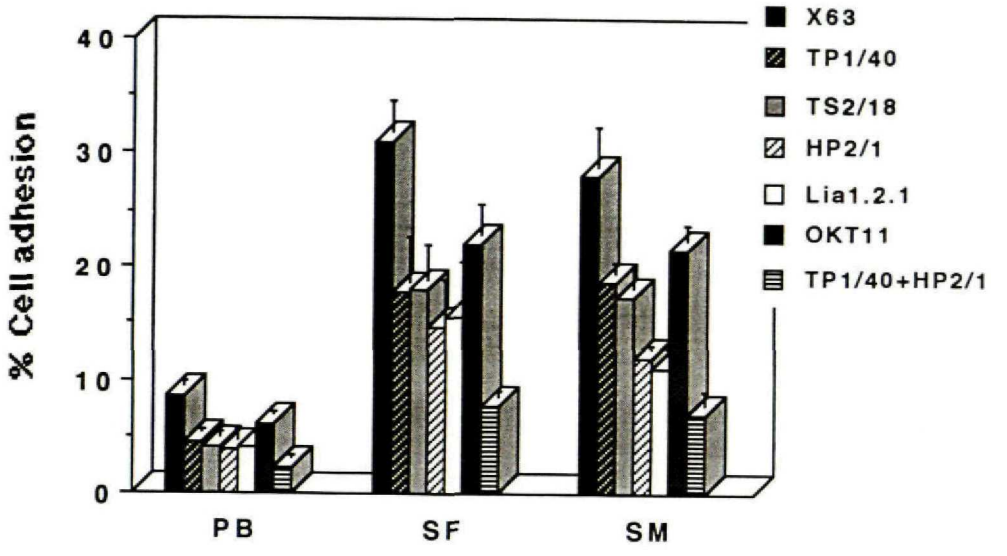

Figure 3. Effect of MAbs on T lymphocytes from different sources in their adhesion to FLS. T cells isolated from PB, SF and SM were preincubated for 45 min. at 4° with saturating concentrations of the following MAbs: anti-LFA-1α (TP1/40), anti-β2 (TS1/18), anti-VLA-4α (HP2/1), anti-β1 (Lia1.2.1), anti-CD2 (OKT11). Adhesion data are referred to the attachment obtained with preincubation of T cells with a control MAb X63. Results are expressed as the mean±SE from 7 (PB and SF) and 4 (SM) separate experiments (see text for values of statistcal significance).

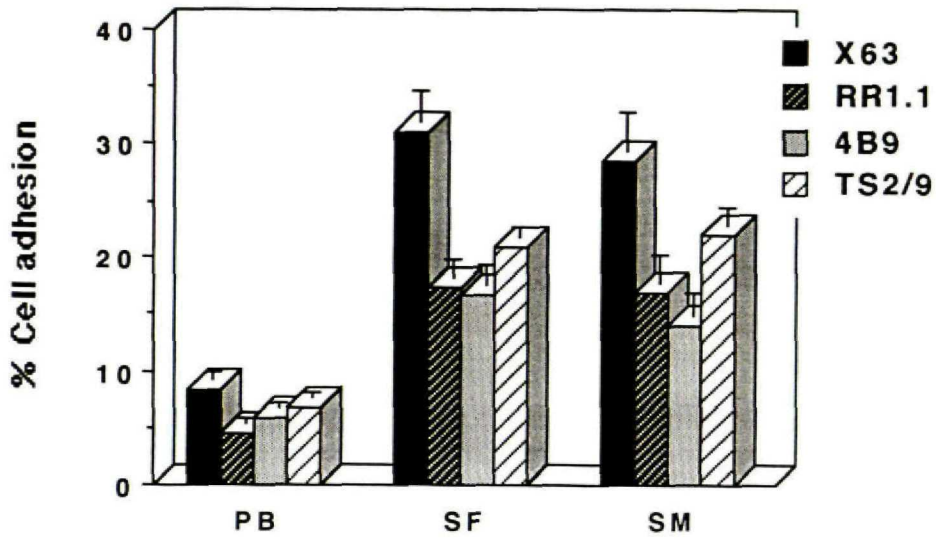

Figure 4. Effect of MAb against FLS molecules in the adhesion of T lymphocytes from different sources. FLS were preincubated in RPMI 10% FCS for 30 min. at room temperature with different MAbs: anti-ICAM-1 (RR1/1.1), anti-VCAM-1 (4B9) and anti-LFA-3 (TS2/7). Adhesion data are referred to the attachment obtained with preincubation of FLS with a control MAb X63. Results are expressed as mean±SE of 7 (PB and SF) or 4 (SM) independent experiments (see text for values of statistical significance). Non significant differences in the effect of MAb were detected when adhesion was performed with RA or control PB T cells.

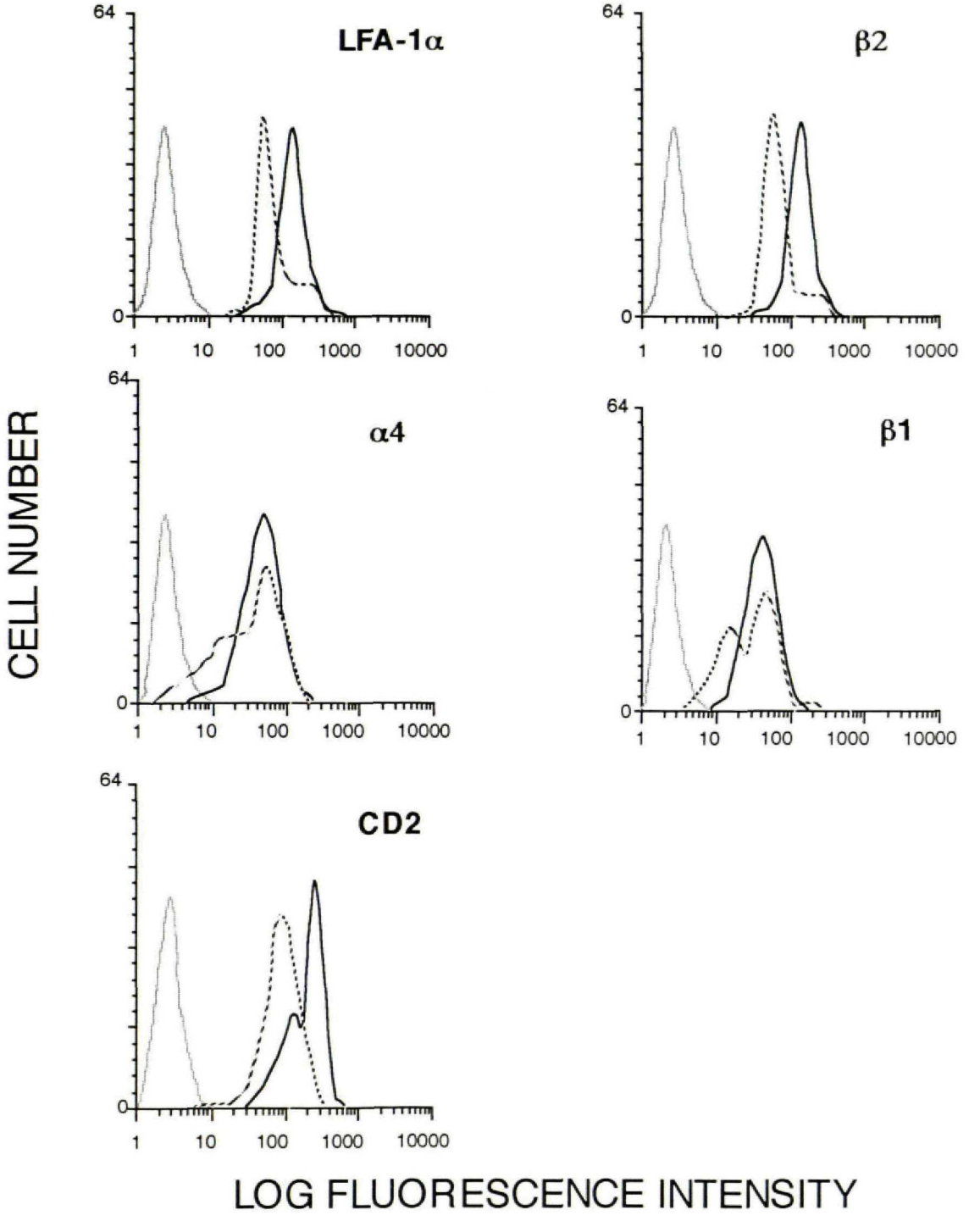

Figure 5. Expression of adhesion molecules on PB and SF T lymphocytes. Cells isolated from PB (striped line), and SF (solid line) were labeled with TP1/40 (anti-LFA-1α), TS1/18 (anti-β2), HP2/1 (anti-α4), Lia1.2.1 (anti-β1) and OKT11 (anti-CD2) MAbs, and analyzed by flow cytometry. Histogram profiles from a representative patient with Rheumatoid Arthritis are shown. Background staining given by the negative control P3X63 MAb is included (dotted line).

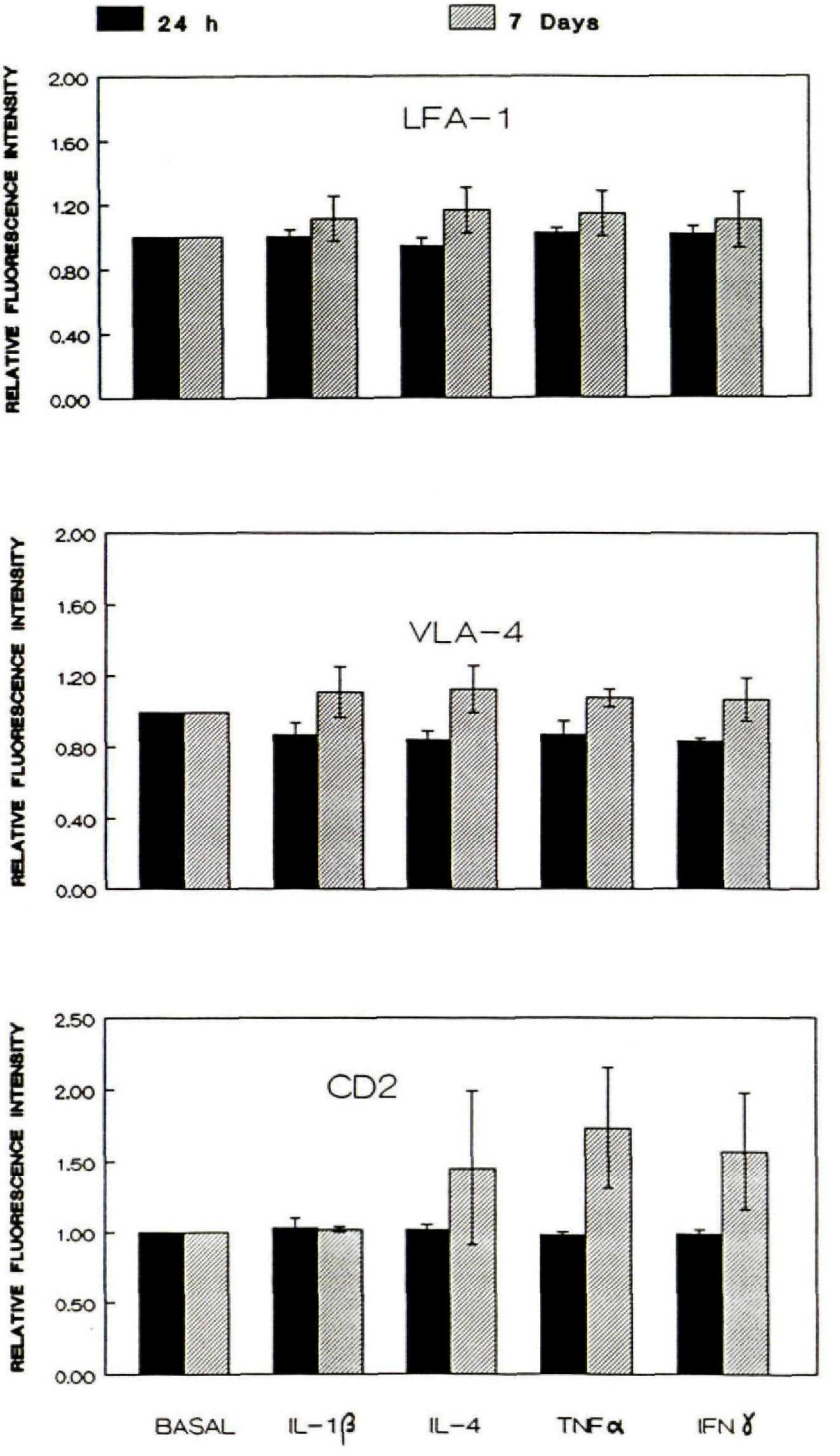

Figure 6. Cytokine modulation of adhesion molecule expression on T lymphocytes. T cells were analyzed by flow cytometry after incubation with cytokines for the times indicated. Mean fluorescence intensity values were obtained in a linear scale. Changes in molecule expression were expressed as mean relative fluorescence: rMFI=absolute $MFI_{cytokine}$/absolute MFI_{medium}

CAPITULO V

REGULACION DE LA EXPRESION E IMPORTANCIA FUNCIONAL DE LA PRESENCIA DE UN EPITOPO CONFORMACIONAL ACTIVADO DE LAS INTEGRINAS β1 EN ARTROPATIAS INFLAMATORIAS CRONICAS

La afinidad de las integrinas VLA por sus ligandos puede ser aumentada por su transición a un estado conformacional activado. Este cambio conformacional puede ser detectado por un nuevo AcM, denominado 15/7, que reconoce un epítopo conformacional activado en la subunidad β1 común de diferentes integrinas VLAαβ1. Para comprender la importancia funcional de la aparición *in vivo* de este de estado conformacional comenzamos investigando la expresión del epítopo 15/7 en células T obtenidas de pacientes con AR y otras artropatías inflamatorias crónicas. La expresión del epítopo 15/7 fué significativamente superior en los linfocitos T del LS respecto a los de SP (Fig. 1 y 2). Posteriormente se analizó el efecto de diferentes citoquinas en la aparición del epítopo activado en células T de SP, donde habitualmente no se detecta. IFNγ, IL-2 y en menor medida TNFα fueron capaces de inducir o incrementar la expresión de esta conformación activada en las células T de SP (Fig 3). Esta expresión incrementada de 15/7 se correlaciona con una mayor capacidad de adhesión a FN de las células T estimuladas con citoquinas (Fig 4). En estudios inmunohistoquímicos, este epítopo de activación se detecta en una pequeña proporción de linfocitos T de infiltrados inflamatorios de MS de AR, glándula tiroidea de tiroiditis de Hashimoto y glándula salivar de S Sjögren, así como en células endoteliales y células del *lining* sinovial (Fig 5 y datos no mostrados). Se investigaron a continuación las posibles consecuencias funcionales de la expresión del epítopo 15/7 en la adhesión celular *in vitro*. Los estudios de inmunofluorescencia mostraron una distribución punteada del epítopo activado selectivamente localizados en los contactos de unión de células mielomonocíticas U-937 adheridas a un sustrato de FN de 80kD (Fig 7). Paralelamente el AcM 15/7 fué capaz de inducir tanto el *despliegue (spreading)* celular como la adhesión de linfocitos T, células Jurkat y U-937 a FN (Fig. 6A y 6B). Estos resultados confirman la presencia *in vivo* de conformaciones activas de integrinas β1 en infiltrados inflamatorios crónicos. Estas conformaciones activas de las integrinas β1 pueden ser reguladas por mediadores

fisiológicos como citoquinas y desempeñan un papel importante en la adhesión y cambios de morfología celular por lo que podrían contribuir decisivamente a la formación del foco inflamatorio.

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The contribution to this paper by A.G.A. and R. G-V is equal and the order of authorship is arbitrary

Expression and functional significance of an activation-dependent epitope of the β1 integrins in chronic inflammatory diseases

The avidity of VLA integrins for their ligands can be increased by their transition to an active conformational state. This conformational change can be detected with a novel monoclonal antibody (mAb), termed 15/7, that recognizes an activation-dependent conformational epitope on the common β1 polypeptide of different VLA αβ1 integrins. In an attempt to understand the possible role of the active conformational state of β1 integrins *in vivo*, we first investigated the expression of 15/7 epitope on T lymphocytes from patients with chronic inflammatory joint diseases. An enhanced expression of the 15/7 epitope was found in the synovial fluid (SF) T lymphocytes from these patients as compared to their peripheral blood (PB) T cells. The effect of different cytokines on the appearance of the 15/7 activation epitope in PB T lymphocytes was subsequently analyzed; interferon-γ, interleukin-2 and, to a lower extent, tumor necrosis factor-α were able to induce an increased expression of the 15/7 epitope. This enhanced 15/7 expression correlated with a higher binding ability to fibronectin of cytokine-activated T cells. The presence of this activation epitope was detected in a small proportion of T lymphocytes scattered within inflammatory foci of synovial membrane from rheumatoid arthritis and thyroid glands from Hashimoto's chronic thyroiditis. We then analyzed the possible role of 15/7 epitope expression on cell adhesion *in vitro*. Immunofluorescence studies showed that the 15/7 epitope displayed a spot-like distribution, selectively decorating adhesive contacts of U-937 myelomonocytic cells attached to the 80 kDa proteolytic fragment of fibronectin (FN80). Furthermore, the anti-β1 15/7 mAb was able to induce both T lymphocyte, Jurkat and U-937 cellular binding and spreading on FN80. Altogether these results indicate that an activated conformation of β1 integrins is detected *in vivo* in lymphocyte infiltrates from chronic inflammatory conditions. The active conformations of β1 integrins are regulated by physiologic mediators such as cytokines, play an important role in cellular attachment and spreading, and appear to be involved in the development of inflammatory processes.

1 Introduction

Cell communication through different adhesion molecules is an essential event in inflammatory responses [1–3]. One of the most important families of such receptors are integrins, that are noncovalently associated αβ heterodimers [4]. The VLA proteins or β1 integrins comprise, at least, nine different αβ1 heterodimers, that are involved in cell-cell interactions and also act as receptors for several extracellular matrix (ECM) proteins [4–7].

Cellular adhesive properties are regulated through the selective expression of the integrin repertoire as well as by the modulation of the binding properties of these receptors. The avidity of integrins for their ligands is not constant and is dynamically regulated. The up-regulation of integrin avidity can be accomplished *in vitro* by treatment of either cells or the isolated integrin with activating stimuli including phorbol esters, physiologic ligands, divalent cations, or mAb [4, 8, 9]. This active conformation of integrins results in an enhanced binding to their ligands.

In some pathologic conditions, an up-regulated integrin function appears to be closely related with the activated state of lymphoid cells [10–13]. This appears to be the case of rheumatoid arthritis (RA), a systemic and chronic inflammatory disease, that affects predominantly joints. The rheumatoid synovial membrane (SM) exhibits a profuse mononuclear cell infiltrate and is one of the best characterized tissues regarding expression of adhesion molecules [14–16]. The predominant infiltrating T cell population, which notably expresses β1 and several α integrins subunits [11, 16], also expresses cell surface activation antigens [10, 17, 18]. This activated cell phenotype, which is shared by synovial fluid (SF) T lymphocytes, contrasts with the predominant resting state of the cells in peripheral blood (PB). Accordingly, rheumatoid synovial T cells exhibit a higher avidity than PB T cells for VCAM-1, the endothelial cell ligand of α4β1 and α4β7 integrins, as well as to several ECM proteins [10, 13, 19, 20]. Since enhanced cell binding cannot be solely explained by a quantitative change in integrin expression, the increased adhesiveness of synovial T cells for different integrin ligands could be due to conformational changes undergone by integrins. Furthermore, the interaction of T cells with all these ligands, as

[I 14189]

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consequence of the increased avidity of their integrin receptors, could drive them to proliferate as well as to change cellular morphology [14, 19, 21, 22]. Interestingly, mAb that recognize activation-dependent epitopes on β1, β2 and β3 integrins have been described [23–27].

In this report, we explored the expression and regulation of different active VLA-integrin conformations by using the mAb 15/7, that recognizes an activation-dependent epitope on the β1 chain. Putative relevant consequences of *in vivo* appearance of this conformational state of β1 integrins in several inflammatory processes were also addressed.

2 Materials and methods

2.1 Patients

This study included patients with RA ($n = 6$) that satisfied The American College of Rheumatology (formely American Rheumatism Association) criteria for the diagnosis of RA [28]. Patients with other chronic inflammatory joint diseases such as ankylosing spondilytis ($n = 1$), psoriatic arthritis ($n = 3$), and oligoarthritis in the context of Behçet's syndrome ($n = 1$) were also studied. Five patients were males and four females, and their mean age was 38.7 years (range 26–59). Three patients were receiving i.m. gold salts, three oral methotrexate, and four were under low-dose prednisone therapy. Six patients were taken nonsteroidal antiinflammatory drugs. After consent, paired samples of SF and blood were obtained from each patient. Blood was also collected from 12 healthy volunteers.

2.2 Cells and tissues

PB and SF mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. Adherent cells were removed by incubation on plastic flasks (Costar Corp., Cambridge, MA). Purification of T cells was achieved by passage through nylon wool columns. The purity of the T cells obtained was >95% as assessed by CD3 expression, <2% CD11b, and <2% CD19. Jurkat T leukemic and myelomonocytic U-937 cell lines were grown in RPMI 1640 medium (Whittaker) supplemented with 5% plus glutamine and antibiotics.

Synovial membrane (SM) specimens were obtained from RA patients that underwent a therapeutic surgery. Thyroid specimens from Hashimoto's thyroiditis were obtained from patients who had undergone therapeutic surgery. All tissues were snap frozen in liquid nitrogen and OCT compound (Ames Company, Miles Laboratories, Elkhart, IN) and stored at −80°C.

2.3 Immunoprecipitation

T lymphocytes were radioiodinated in solution with chloroglycoluril (IODO-GEN, Pierce, Chem. C., Rockford, IL), lysed and immunoprecipitated with different mAb. The immune complexes were isolated by incubation with 187.1 anti-mouse κ chain mAb followed by protein A-Sepharose. The samples were processed and subjected to SDS-10% PAGE under reducing conditions.

2.4 Reagents, cytokines and monoclonal antibodies

Phorbol myristate acetate (PMA) was purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Recombinant human (h)IL-1β (specific activity 5×10^8 U/mg, purity >95%, LPS content <0.015 ng/ml) was purchased from Amgen Biologicals (Thousand Oaks, CA). Recombinant hTNF-α (spec. act. 5×10^7 U/mg, purity >95%) was provided by Genentech (South San Francisco, CA). Recombinant hIFN-γ (spec. act. 2×10^7 U/mg, purity >98%, LPS content <0.048 ng/ml) was a gift from Amgen Biological. Recombinant hTGF-β (purity >97%, LPS <0.02 ng/ml) was purchased from R & D Systems, Inc. (Minneapolis, MN). Recombinant IL-4 (specific activity 2×10^7 U/ml) was purchased from Promega (Madison, WI). In each case, the final concentration of LPS in culture after dilution of recombinant cytokine was <0.005 ng/ml.

15/7 mAb reacts with an activation epitope on human β1 integrin [23]. The 15/7 mAb recognizes β1 integrin by flow cytometry, Western blot, and immunoprecipitation analysis. This epitope can be induced on β1 integrin-positive cells by distinct activating agents including PMA, Mn^{2+}, and the activating anti-β1 antibodies TS2/16 and 8A2 ([23], Yednock et al., unpublished observations). The anti-β1 TS2/16 mAb has previously been described [29]. The anti-ICAM-3 TP1/25, and anti-CD69 TP1/8 mAb have already been reported [30–32]. The anti-CD3 mAb was purchased from Dakopatts (Glostrup, Denmark).

2.5 Cytokine treatment of T cells

PB T cells were cultured for 24–72 h in RPMI supplemented with 10% FCS (Whittaker), 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine in a humidified 5% CO_2 atmosphere in presence or absence of each cytokine: IL-1β (100 U/ml), IL-4 (100 U/ml), TNF-α (1000 U/ml) IFN-γ (100 U/ml), and TGF-β (10 ng/ml). PB T cells were incubated with phytohaemagglutinin (5 μg/ml) for 48 h before treatment with IL-2 (50 U/ml).

2.6 Flow cytometric analysis

Fluorescence flow cytometry analysis was performed on a FACScan cytofluorometer (Becton Dickinson, Mountain View, CA). Cells were incubated at 4°C with either 20 μg/ml 15/7 mAb or 100 μl hybridoma culture supernatant TS2/16 mAb, followed by washing and labeling with FITC-conjugated goat anti-mouse Ig (Dakopatts). Data were collected both in linear and logarithmic scales. Five thousand cells were analyzed from each sample. In every experiment, the sample with the greatest fluorescent intensity was analyzed first, and the fluorescent gain was adjusted so that 1–5% of cells were positive in the highest fluorescent channel. A negative control was then analyzed, and the upper limit of negativity was established by determining the lowest channel that would include 95–99% of negative control cells. Because the fluorescence conditions were different from experiment to experiment, data were nor-

malized to express relative fluorescence intensity (rMFI) as follows: rMFI = absolute MFI cytokine/absolute MFI medium after substracting from each MFI the MFI of the negative control (P3X63). The % Δ MFI was obtained as follows: % Δ MFI = (absolute MFI cytokine/absolute MFI medium) - 1 × 100.

2.7 Cell attachment assays

The attachment of U-937, Jurkat cells, and peripheral blood T lymphocytes to a proteolytic fragment of fibronectin, FN80, was assayed as previously described [33]. Briefly, 96-well flat-bottom plates (Titertek, Flow Laboratories, Irvine, Scotland) were coated overnight at 4 °C with FN80 (10 μg/ml) diluted in phosphate-buffered saline (PBS). Thereafter, the plates were saturated with 1 % BSA 1 h at 37 °C. Plates were washed twice with PBS and cells (preincubated with different mAb or medium as control) were added in serum free medium and incubated for 30 min at 37 °C. To quantify cell attachment, the plates were washed twice with RPMI, cells were fixed with a mixture of acetone/methanol 1 : 1 and dyed with violet crystal 0.5 %. Then, absorbance at 540 nm was measured in an ELISA reader (LP400, Kallestad, Chaska, MN). A calibration curve was made for each cell type and optical density was found to be a linear function of number of cells. Basal adherence to BSA-coated wells was subtracted from attachment values obtained. All assays were performed in triplicate and results were expressed as % of specific adherence.

2.8 Immunofluorescence studies

Glass coverslips were coated overnight at 4 °C with FN80 at 10 μg/ml diluted in PBS. The coated coverslips were rinsed in PBS, saturated with 1 % BSA for 1 h at 37 °C and rinsed again. After the induction of cell adhesion at 37 °C for 30 min to the substratum-coated coverslips with either anti-β1 TS2/16 (1 μg/ml) or 15/7 mAb (7 μg/ml), cells were fixed in 3.7 % formaldehyde in PBS for 10 min at room temperature and rinsed in TBS (TBS: 50 mM Tris-HCl, 150 mM NaCl, 0.1 % NaN_3, pH 7.6). Cells were stained for β1 integrin with biotinylated 15/7 or TS2/16 mAb for 1 h and rinsed in TBS. Then, cells were incubated with an 1 : 1,000 dilution of TRITC-avidin D (Vector, Burlingame, CA), then with an 1 : 100 dilution of anti-avidin D-biotin (Vector), and again with an 1 : 1,000 dilution of TRITC-avidin D. Coverslips were washed in TBS, rinsed in H_2O, and mounted with Mowiol (Calbiochem). The samples were observed using a Nikon Labophot-2 photomicroscope with a 60 × oil immersion objective and photographed on TMAX 400 film (Eastman Kodak Co., Rochester, NY) processed to 800–1600 ASA with TMAX developer (Eastman Kodak Co.).

2.9 Immunohistochemical staining of human tissue sections

Acetone-fixed 4-μm-thick frozen sections were incubated with mAb culture supernatants for 30 min at room temperature. Subsequently, sections were incubated with a peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts). Each incubation was followed by three washed with Tris saline (TBS) isotonic buffer (pH 7.6). Then, sections were developed with the Graham-Karnovsky solution containing 3,3′-diaminobenzidine (DAB) hydrochloride (0.5 mg/ml) and hydrogen peroxide. The reaction was stopped by washing with TBS, and sections were counter-stained with Carazzi's hematoxylin, dehydrated, and mounted by routine methods.

In double immunostaining, after the development of the DAB reaction, the sections were saturated with non-specific mouse Ig, washed and then incubated with the second mAb in the same conditions. Subsequently, they were incubated with a rabbit anti-mouse IgG alkaline phosphatase complex (Dakopatts). Each incubation was followed by three washes with TBS. Finally, the alkaline phosphatase reaction was developed by incubating the sections with a solution of 0.2 mg/ml of Naphtol AS-MX phosphate, 1 mg/ml of Fast Blue salt and 10^{-5} M Levamisole (all from Sigma Chem. Co.) dissolved in 50 mM Tris HCl, pH 8.4. The reaction gives a bright blue precipitate that contrasts with the brown colour of the DAB reaction. Sections were mounted in buffered gelatin for microscope examination.

2.10 Statistical analysis

To determine the significance of differences, we used the non-parametric Mann-Whitney U test.

3 Results

3.1 Up-regulated expression of β1 activation epitope in SF lymphocytes from chronic inflammatory joint diseases

The β1 activation epitope recognized by 15/7 mAb is hardly detected in resting PB lymphocytes (Fig. 1 A). This epitope is clearly expressed on cells treated with stimuli that activate β1 integrins, such as Mn^{2+} plus the regulatory anti-β1 mAb TS2/16 (Fig. 1 A). We studied the expression of the 15/7 β1 activation epitope on cells which are activated *in vivo*, as T lymphocytes infiltrating the rheumatoid synovium. The expression of this epitope was clearly detected on human SF T lymphocytes, but it was virtually absent on PB T cells, as illustrated in a representative patient with RA (Fig. 1 B). Quantitative estimation of the expression of the 15/7 epitope on T lymphocytes from PB and SF patients with various chronic inflammatory joint diseases (RA, psoriatic arthritis, ankylosing spondylitis), revealed significant higher percent of cells bearing 15/7 epitope in SF compared to PB T cells from the same patients (Fig. 2 A). Similar data were obtained by cytofluorometric analysis of T cells isolated from an RA synovial membrane (data not shown). No difference in 15/7 mAb expression was observed between PB T cells from control healthy donors and RA patients (Fig. 2 A). Moreover, an increase in the 15/7 epitope density expression (fluorescence intensity) was also detected in SF T cells (Fig. 2 B). The expression of this epitope by SF T cells was further enhanced by *in vitro* treatment with the integrin activatory stimuli Mn^{2+} plus the anti-β1 TS2/16 mAb (data not shown).

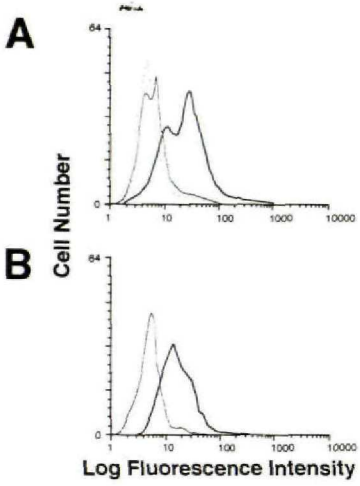

Figure 1. *In vitro* and *in vivo* expression of the 15/7 β1 activation epitope. (A) Peripheral blood lymphocytes either untreated (...) or treated with 1 mM Mn^{2+} plus 1 μg/ml TS2/16 F(ab')$_2$ mAb (——) were analyzed for 15/7 epitope expression by flow cytometry as described in Sect. 2.6. A representative experiment out of three independent ones is shown. (B) T cells from SF (——) or PB (...) from a patient with RA were labeled with the 15/7 mAb. Data were collected in a logarithmic scale. Background staining given by the negative control mouse myeloma P3X63 is included (- - -).

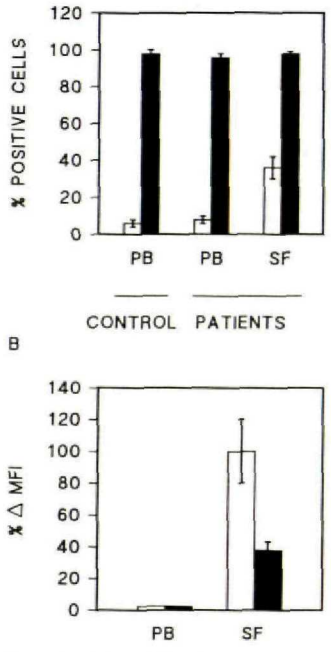

Figure 2. The 15/7 epitope is induced *in vivo* in chronic arthritis. SF and PB T cells from nine patients with chronic inflammatory arthritis were analyzed for the expression of epitopes recognized by the anti-β1 15/7 and TS2/16 mAb by flow cytometry as described in Sect. 2.6. Data were collected on a linear scale, and the % positive cells (A) as well as the % increment in the MFI of SF T cells (B) were determined. Absolute MFI of PB T cells was arbitrarily assigned the value 1. Arithmethic mean ± 1 SD from the nine different patients is represented. 15/7 and TS2/16 expression in PB T cells from healthy donors (control) is also included (2A). □ 15/7, ■ TS2/16.

Altogether these data indicate that β1 integrin receptors on T lymphocytes acquire a differently active conformation during *in vivo* T cell activation, which is detectable with the 15/7-specific mAb.

3.2 Cytokines regulate the expression of the β1 activation epitope

Several cytokines including IL-1, TNF-α, IFN-γ, granulocyte-macrophage (GM)-CSF, IL-4, IL-6, IL-8 and transforming growth factor (TGF)-β, have been implicated in the pathogenesis of RA [34–38]. It is well known that these cytokines are able to induce the *de novo* synthesis as well as increase the expression of some adhesion molecules [39]. Therefore, we analyzed the effect of different cytokines on the expression of the 15/7 epitope by T lymphocytes from healthy donors. PMA was also included as positive control of cell stimulation.

The treatment of T cells with IFN-γ was able to increase significantly the expression of the 15/7 epitope in five out of seven experiments (Fig. 3A, Table 1). This up-regulation took over 24 h and reached a plateau at 72 h. TNF-α induced a slight increase of 15/7 expression on T lymphocytes, which became significant after prolonged stimulation (Fig. 3A, Table 1). IL-2 was also able to induce an enhancement in the expression of 15/7 epitope at 24 h in two out of three experiments (13 % and 39 %), and at 72 h in the third one (24 %). No significant effect in 15/7 expression was observed when T cells were pretreated only with PHA (data not shown). Other cytokines including IL-1β, IL-4, IL-10, and TGF-β did not induce the appearance of the 15/7 epitope (data not shown). PMA increased the expression of the 15/7 activation epitope in all cases studied with optimal stimulatory effect at 72 h (Fig. 3A).

Table 1. Cytokine modulation of 15/7 epitope expression by PB cells[a]

	Percentage of positive cells			
mAb	Anti-β1 activation epitope (15/7)		Anti β1 (TS2/16)	
	Time (h)			
Cytokine	24 h	72 h	24 h	72 h
Medium alone	5 ± 1	7 ± 1	86 ± 6	90 ± 4
TNF-α (1000 U/ml)	8 ± 1	14 ± 1*	77 ± 14	96 ± 3
IFN-γ (100 U/ml)	18 ± 5*	20 ± 2*	91 ± 3	95 ± 2
PMA (20 ng/ml)	25 ± 6*	39 ± 6*	95 ± 1	99 ± 1

a) Cells were preincubated with each cytokine for the times indicated. Results are expressed as mean ± SE in terms of percentages of positive cells. Data were obtained by substracting the number of background cells that were non specifically stained with the control mAb X63. Statistical significance: * $p < 0.05$ compared to medium alone.

TNF-α and PMA had an inhibitory effect on the expression (MFI) of β1 integrins (detected with the TS2/16 mAb) at 24 h. However, after 72 h the level of β1 expression was only slightly affected by TNF-α and increased by PMA treatment (Fig. 3B). IFN-γ treatment increased the expression of β1 integrins at both 24 and 72 h (Fig. 3B).

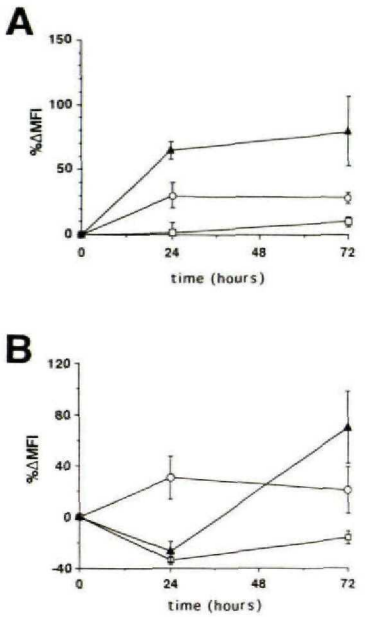

Figure 3. Cytokines regulate the expression of the 15/7 β1 epitope in T cells. PB T cells were incubated with the cytokines indicated for different times, and then analyzed for the expression of epitopes recognized by the 15/7 or TS2/16 mAb (A and B, respectively) by flow cytometry as described in Sect. 2.6. The % of increment of the mean of fluorescence intensity is represented. Arithmethic mean ± 1 SE from at least four experiments is shown. □ TNF-α, ○ IFN-γ, ▲ PMA.

TNF-α and IFN-γ did not have a significant effect on the percent of cells bearing the TS2/16 epitope (Table 1). The induced 15/7 expression correlated with an increased binding ability of cytokine-treated T cells to fibronectin. As shown in Fig. 4, the adhesion of PB T cells to FN80 was closely related to the level of expression of the 15/7 β1 activation epitope induced by IFN-γ or PMA treatment.

Altogether, these results indicate that IFN-γ, IL-2, and TNF-α can regulate the *in vitro* appearance of the 15/7 β1 activation epitope, and suggest that these cytokines have

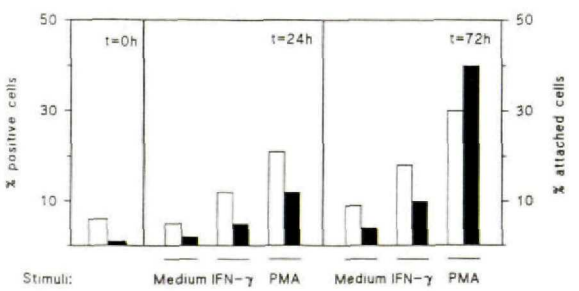

Figure 4. Enhanced 15/7 expression correlated with the binding ability of cytokine-treated PB T cells to fibronectin. PB T cells were incubated with IFN-γ or PMA for different times, and then analyzed for either the expression of the 15/7 epitope (□) or the adhesion to fibronectin (■) as described in Sect. 2.7. Results are represented as % positive cells for 15/7 expression and % attached cells for adhesion to FN. A representative experiment out of two is shown. SD was less than 10% of the mean of adhesion data.

an important role in the *in vivo* regulation of the active conformation of β1 integrins in inflammatory conditions.

3.3 Expression of the β1 activation epitope in tissue sections from various inflammatory diseases

To investigate the pattern of cellular and tissue distribution of β1 activation epitope, immunohistochemical staining of tissue sections from different chronic inflammatory diseases was carried out. A subset of mononuclear cells within inflammatory infiltrates were found positive for the expression of the 15/7 epitope in synovial membranes from RA, thyroid glands from Hashimoto's chronic thyroiditis (Fig. 5, A and D, respectively), and salivary glands from Sjögren's syndrome (data not shown). Most of these 15/7-positive cells were demonstrated to be T lymphocytes as assessed by parallel as well as double-staining analysis with anti-CD3 (T cell specific marker) and 15/7 mAb (Fig. 5, C and F). The 15/7-positive cells were mainly located in close proximity to vessels. Additional reactivity was detected on larger mononuclear cells, likely macrophages, which were scattered into the interstitial space. Endothelial cells also showed a strong reactivity with the 15/7 mAb (Fig. 5, A and D). The pattern of 15/7 staining was different from that obtained with the anti-β1 TS2/16 mAb; a lower proportion of cells present in tissue specimens from the different diseases reacted with 15/7 mAb compared with the TS2/16 mAb (compare Fig. 5, A and D to 5, B and E).

These data indicate that lymphocytes bearing the active conformational state of β1 integrins are preferentially localized at sites of tissue injury in different chronic inflammatory processes.

3.4 Role of 15/7 activation epitope on cell attachment and spreading on fibronectin

To assess the functional consequences of 15/7 cell expression, cell attachment assays with PB T lymphocytes, Jurkat T leukemic, and U-937 myelomonocytic cells were performed. FN80, that contains the binding site of the VLA-5 integrin, was used, and assays were performed either in the presence or the absence of the 15/7 mAb. Interestingly, the anti-β1 mAb was able to enhance significantly the specific attachment of the three cellular types to FN80 (Fig. 6a). Other stimuli such as the activating anti-β1 TS2/16 mAb, Mn^{2+}, and PMA were included as controls.

We next investigated the effect of the 15/7 mAb on cellular morphology by immunofluorescence. Both Jurkat and U-937 cells were pretreated with the 15/7 mAb, allowed to attach to FN80, and then labeled with anti-β1 TS2/16 mAb. 15/7 mAb induced the appearance of thin cellular projections, termed filopodia (Fig. 6b, B and D), that were not observed in untreated cells (Fig. 6b, A and C).

Finally, we performed cell localization studies of the 15/7 β1 activation epitope on cells bound to ECM proteins. To this end, U-937 cells treated with the TS2/16 mAb were allowed to attach to FN80, and then, labeled by immunofluorescence with the 15/7 mAb. The β1 activated epitope

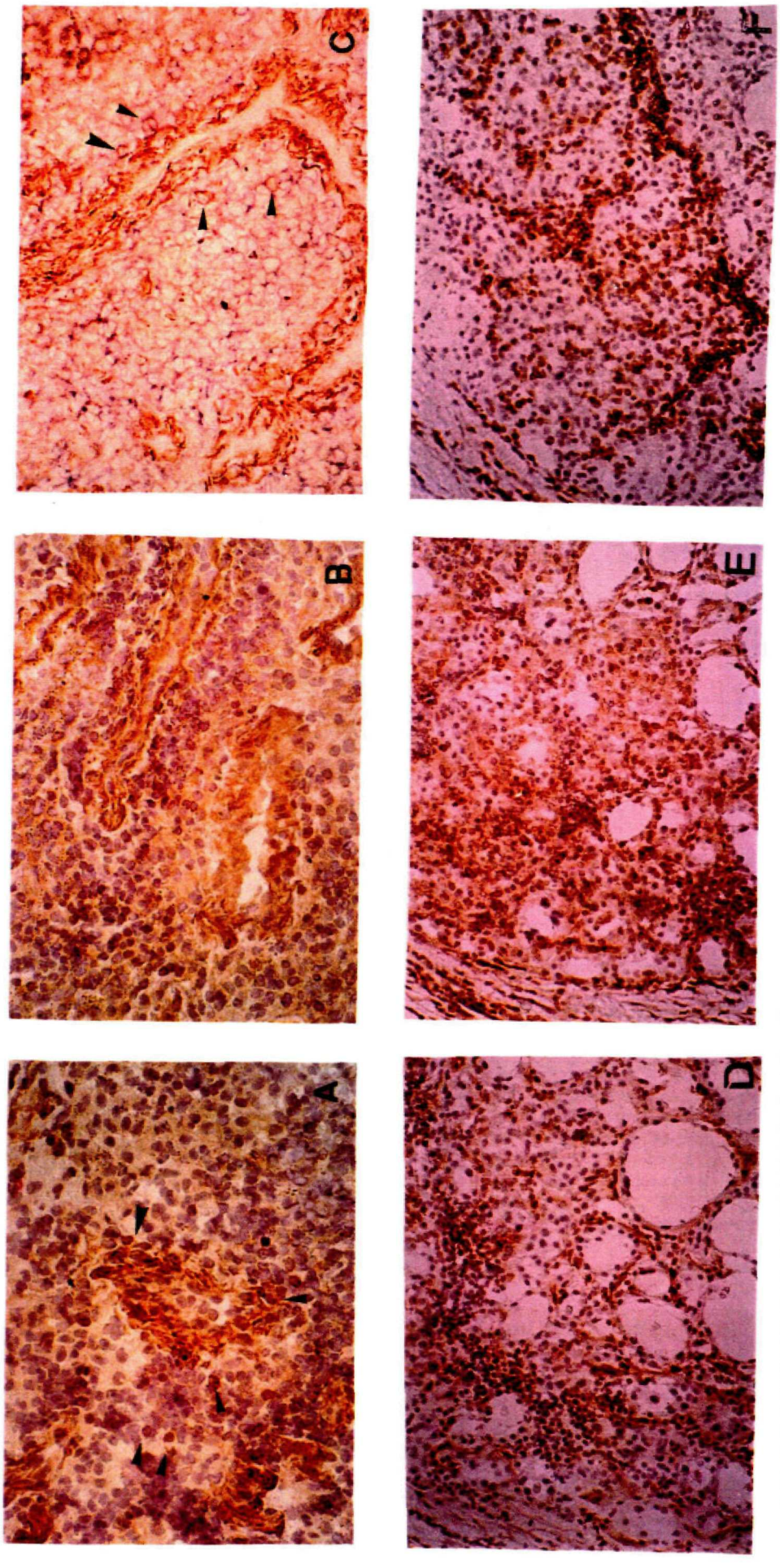

Figure 5. Tissue expression of 15/7 β1 epitope in different chronic inflammatory diseases. The expression of 15/7 epitope was determined by immunohistochemical analysis in tissue specimens from different chronic inflammatory diseases including synovial membrane from RA and thyroid gland from Hashimoto's chronic thyroiditis. RA sections were stained with 15/7 anti-β1 (A), TS2/16 anti-β1 (B), and double stained with the anti-CD3 (alkaline phosphatase) and 15/7 (peroxidase) (C). Arrows indicate double stained cells. Serial sections of Hashimoto's thyroiditis were stained with 15/7 anti-β1 (D), TS2/16 anti-β1 (E), and anti-CD3 (F). Magnifications were x 500 (A, B, C), and x 250 (D, E, F).

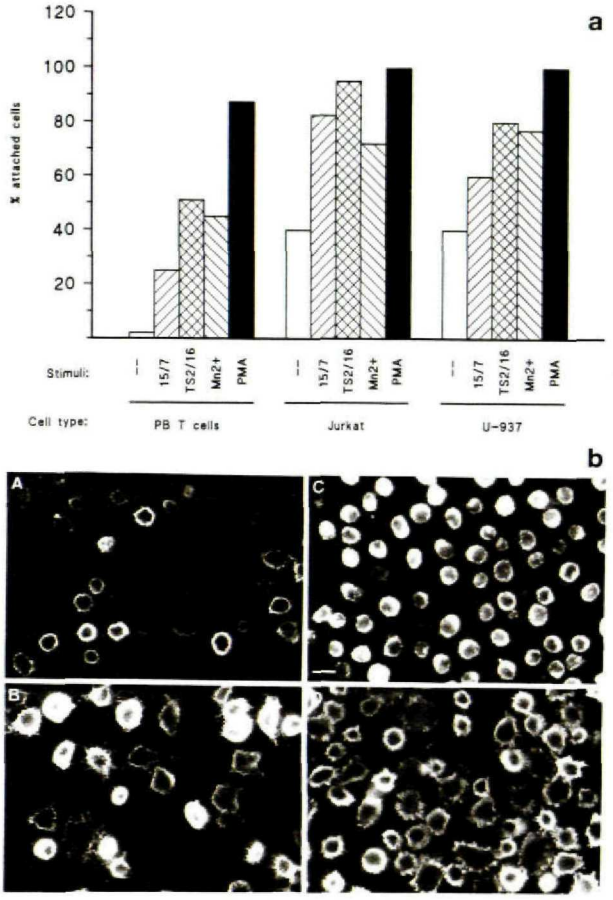

Figure 6. Effect of 15/7 mAb on cellular adhesion and spreading. (a) Cell binding assays. PB T lymphocytes, Jurkat or U-937 cells either untreated or treated with the 15/7 mAb were assayed for attachment to FN80-coated plates for 30 min, and then, cell binding was quantified as described. A representative experiment out of three independent ones is shown. Assays were performed by triplicate. SD of the mean was less than 10%. Results with other stimuli (TS2/16, Mn^{2+}, PMA) are included as controls. (b) Cellular spreading. Jurkat or U-937 cells either untreated (A and C) or treated (B and D) with the 15/7 mAb were assayed for attachment to FN80-coated slides for 30 min, and then, labeled with the anti-β1 TS2/16 mAb. Note the appearance of thin projections when cells were treated with the 15/7 mAb. Bar, 60 μm.

displayed a spot-like distribution along adhesive contacts in cells stimulated with TS2/16 (Fig. 7B). Some specific labeling was also detected in unstimulated U-937 cells (Fig. 7A). Similar staining pattern but weaker was obtained using Jurkat T cell line (data not shown).

These results suggest that the active conformational state of β1 integrins is related to the ability of cells to attach and spread on ECM components.

4 Discussion

In this report, we describe the expression of activated conformations of β1 integrins in lymphocytes from the cellular infiltrate of various chronic inflammatory diseases. We

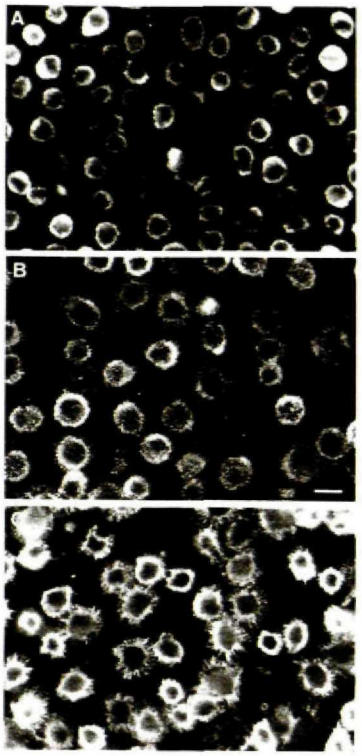

Figure 7. Cellular localization of 15/7 β1 epitope in U-937 cells attached to FN80. U-937 cells were either untreated or treated with anti-β1 TS2/16 mAb (A and B, respectively) before assayed to attach to FN80. Then, the cells were labeled for 15/7 epitope expression. Note the spot-like 15/7 staining along adhesive contacts induced by TS2/16 mAb (B). Cells pretreated and labeled with TS2/16 mAb (C) are included for comparison. Bar, 60 μm.

have also assessed the role of several cytokines in regulating the cell expression of the active conformations of β1 integrins. In addition, we have demonstrated the functional consequences of β1 integrin activation on both cellular adhesiveness and cell morphology.

The existence of different conformations of β1 integrins *in vivo* has been inferred from cell adhesion studies with isolated lymphocytes from inflammatory cell infiltrates. Rheumatoid synovial T cells display a higher affinity than PB T cells for the endothelial cell ligand VCAM-1, as well as for different ECM proteins that are ligands for integrins [10, 11, 13, 19]. However, the direct assessment of the expression of activated β1 integrins *in vivo* had not been explored so far. The 15/7 mAb – which detects an activation-dependent epitope of β1 integrins – makes feasible the study of activated β1 expression both *in vivo* and *in vitro*. The coordinated expression of β1 and β2 integrin activation epitopes during T cell activation in secondary lymphoid tissues has recently been described [23]. However, the expression of β1 activation epitopes during abnormal (autoimmune) conditions had not been so far investigated. Our results, that show an enhanced expression of the 15/7 epitope in cell infiltrates, provide a formal evidence for the presence of cells bearing β1 integrins in active conformation in abnormal autoimmune responses.

It has previously been described the up-regulated expression of adhesion receptors in several cell types from both SF and SM from RA patients [14, 16]. This phenomenon is likely due to increased concentrations of certain cytokines in the synovial microenvironment [40, 41]. TGF-β which is detected in the synovial membrane of RA, is a potent regulator of β1 integrin expression on epithelial and mesenchymal cells [42, 43]. Furthermore, the ability of IL-1β and TNF-α to up-regulate synergistically the expression of the α1 integrin has recently been described [44]. However, little information is available regarding the role of cytokines on the β1 integrin expression by T lymphocytes, as well as on the modulation of integrin affinities for their ligands. Our results on the induction of the 15/7 epitope cell expression by IFN-γ, IL-2 and TNF-α suggest that these cytokines have an important role in the regulation of avidity and, therefore, influence the function of integrins at sites of inflammation. It is feasible that in some conditions, these biological mediators can induce the appearance of 15/7 activation epitope without affecting the density of β1 integrin cell expression. Therefore, the conformational state and the expression degree of β1 integrins might be independently regulated.

Our findings on 15/7 cell expression in various chronic inflammatory diseases prompted us to investigate the possible functional consequences of activated β1 integrin expression on lymphoid cells. Our findings on the selective localization of activated β1 integrins on adhesive contacts suggest that these activated receptors play an important role on cell adhesion. Furthermore, the 15/7 mAb has an enhancing effect on VLA-5-mediated cell attachment to FN80, an effect that has been also found with mAb that recognize constitutively expressed epitopes of β1 integrins, like the one recognized by the TS2/16 mAb [33]. The expression of the 15/7 epitope on cells located near to blood vessels and the correlation found between 15/7 expression and cell adhesion to fibronectin also suggest that this epitope may play an important role in the migration of lymphoid cells to inflammatory foci. The expression of β1 integrins in high-affinity conformations by endothelial cells further supports this point. It is worth mentioning that the tissue distribution studies should be interpreted with caution since the 15/7 epitope is dependent upon the conformation of β1 integrin, and we do not exactly know whether the native configuration of the integrin complex is preserved on fresh frozen tissue sections.

The effect of the 15/7 mAb on cell adhesiveness and morphology suggests that the 15/7 epitope could participate in the triggering of intracellular signals. These signals could be related with the activation of lymphocytes at sites of chronic inflammation, as well as with the release of degradative enzymes and the persistent cytokine gene expression that occur in this condition [14, 45]. The preferential expression of the 15/7 epitope on cell infiltrates from chronic inflammatory diseases suggests that this epitope could be a possible target for immunointervention in these conditions. Interestingly enough, the binding of some mAb to active conformational epitopes on cell adhesion molecules has an inhibitory effect on cellular functions [46, 47]. Future studies on this point appear to be warranted.

In summary, we have provided data on the expression of the β1 integrin activation-dependent epitope in several chronic inflammatory diseases, and on the role of several cytokines in the appearance of this epitope. The functional significance of the 15/7 epitope can be inferred from its pattern of tissue expression and from its involvement in the modulation of both cellular adhesiveness and cell morphology.

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ANEXO (CAPITULO V).

REGULACION POR QUIMIOQUINAS DE LA EXPRESION DE EPITOPOS CONFORMACIONALES ACTIVADOS DE INTEGRINAS β1 EN LINFOCITOS T.

En el presente capítulo hemos demostrado la capacidad de mediadores fisiológicos como las citoquinas TNFα, IFNγ o IL-2, en la inducción o aumento de expresión de conformaciones activas de integrinas β1 en linfocitos T de SP. Las quimioquinas son un subgrupo de citoquinas con capacidad quimiotáctica selectiva para atraer distintos grupos de leucocitos. Las quimioquinas de la subfamilia C-X-C son capaces de actuar sobre diferentes tipos de linfocitos T y se ha descrito su importante función reguladora en la capacidad adhesiva de las integrinas (Springer 1994, Schall y Bacon, 1994). Por lo tanto, nos propusimos investigar la capacidad de las quimioquinas RANTES y MIP1-β en la aparición de epítopos conformacionales activados de integrinas β1 en linfocitos T de SP. Ademas del epítopo reconocido por el AcM 15/7, ya descrito, estudiamos la presencia de otro epítopo dependiente de activación reconocido por el AcM HUTS 21. Este anticuerpo ha sido recientemente descrito dentro de un grupo de AcMs capaces de detectar conformaciones activas de integrinas VLA y que reconocen una nueva región reguladora en la cadena β1 común (Luque et al., 1996).

MATERIAL Y METODOS

Anticuerpos y reactivos. Los AcM P3X63 (control), TS2/16 (anti-β1) y 15/7 (anti-β1 activada) han sido descritos en los capítulos previos de resultados. El AcM HUTS 21 reconoce neoepítopos de activación en la cadena β1 (Luque et al., 1996).

Las quimioquinas humanas recombinantes RANTES (actividad específica 2-5x10^3U/mg, pureza>97%) y MIP1-b (actividad específica 1,6-2,5x10^4U/mg, pureza>97%) fueron suministradas por R&D Systems, Inc (Minneapolis, MN). PMA y $MnCl_2$ fueron adquiridos a Sigma Chemical Co (St Luis, MO).

Células. Los linfocitos T fueron aislados de muestras de SP venosa de 9 voluntarios sanos, según protocolo descrito en anteriores capítulos. Las células T purificadas se suspendieron en RPMI suplementado con 2 mg/ml de D-glucosa e incubadas durante 15 min. a 37°, en atmósfera con 5% CO_2, con RANTES (0,1ng/ml), MIP1-β (0,1ng/ml), $MnCl_2$ (500μM) o PMA (20ng/ml). Posteriormente, los AcMs HUTS 21 (5μg/ml), 15/7 (20μg/ml) y

X63 (1/2 concentración final) se añadieron en presencia de las quimioquinas, PMA o $MnCl_2$, durante otros 15 min a 37°. Tras dos lavados con RPMI a temperatura ambiente, las células fueron teñidas con el segundo anticuerpo conjugado con fluoresceína durante 15 min a 4°, y finalmente lavadas dos veces con PBS a 4°.

Citometría de flujo. El análisis de las células teñidas por inmunofluorescencia indirecta en el citómetro de flujo ha sido descrito en capítulos previos.

RESULTADOS

En consonancia con lo referido previamente para el epítopo reconocido por el AcM 15/7, la expresión del epítopo conformacional activado HUTS 21 en células T en reposo es muy baja y sólo se expresa en el 7,2±4 % (media±ES) de las células . Es bien conocido que la avidez/afinidad de las integrinas por sus ligandos puede ser aumentada *in vitro* por el tratamiento de las células o de la integrina aislada con ésteres de forbol o cationes divalentes (Ver Introducción general epígrafe 3.3.3. y del Capítulo V de Resultados). Cuando las células T fueron tratadas con PMA o Mn^{++} observamos la inducción o aumento de la expresión de la conformación activa que reconocen ambos AcMs, aunque fué más llamativa para HUTS 21 (Fig.1). La tabla 1 recoge los incrementos de intensidad de fluorescencia en la expresión de este epítopo conformacional obtenidos de 6 experimentos independientes.

Como puede observarse en la misma tabla, las quimioquinas RANTES y MIP1β fueron igualmente capaces de aumentar significativamente la expresión de HUTS 21, aunque las respuestas mostraron gran variabilidad dependiendo del donante. Asímismo, estos efectos fueron tiempo y dosis-dependientes, por lo que la dosis y el tiempo de exposición óptimos fueron elegidos tras experimentos previos de tiempo y dosis-respuesta (datos no mostrados). La figura 2 muestra el efecto de ambas quimioquinas en la aparición de los dos epítopos conformacionales activos en un experimento representativo. Este aumento de conformaciones activas no es dependiente de un aumento en el número total de moléculas β1, ya que la expresión de esta integrina detectada por el AcM TS2/16 no se modificó significativamente bajo el efecto de las quimioquinas ni Mn^{++}.

Tabla 1. Expresion del epítopo conformacional activado HUTS 21 de la integrina β1 en linfocitos T de SP bajo diferentes estímulos.

	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6
Medio	1	1	1	1	1	1
PMA (20ng/ml)	3,3	3	ND	4,2	2,8	5
Mn^{++} (500μM)	4	6	13,5	3,3	2,3	3,2
RANTES (0,1ng/ml)	1,6	2,8	3,6	1,6	1,2	1,6
MIP 1β (0,1ng/ml)	1,7	4,3	2,9	4,2	1,2	1,6

Las células T fueron incubadas durante 30 minutos con medio sólo, o en presencia de PMA, Mn^{++}, o quimioquinas, y posteriormente se analizó por citometría de flujo la tinción con el AcM HUTS 21. Los datos se expresan en Intensidad de Fluorescencia relativa respecto a la expresión en condiciones basales (medio) IFR = IF $absoluta_{Estímulo}$/IF $absoluta_{Medio}$. ND: no determinado.

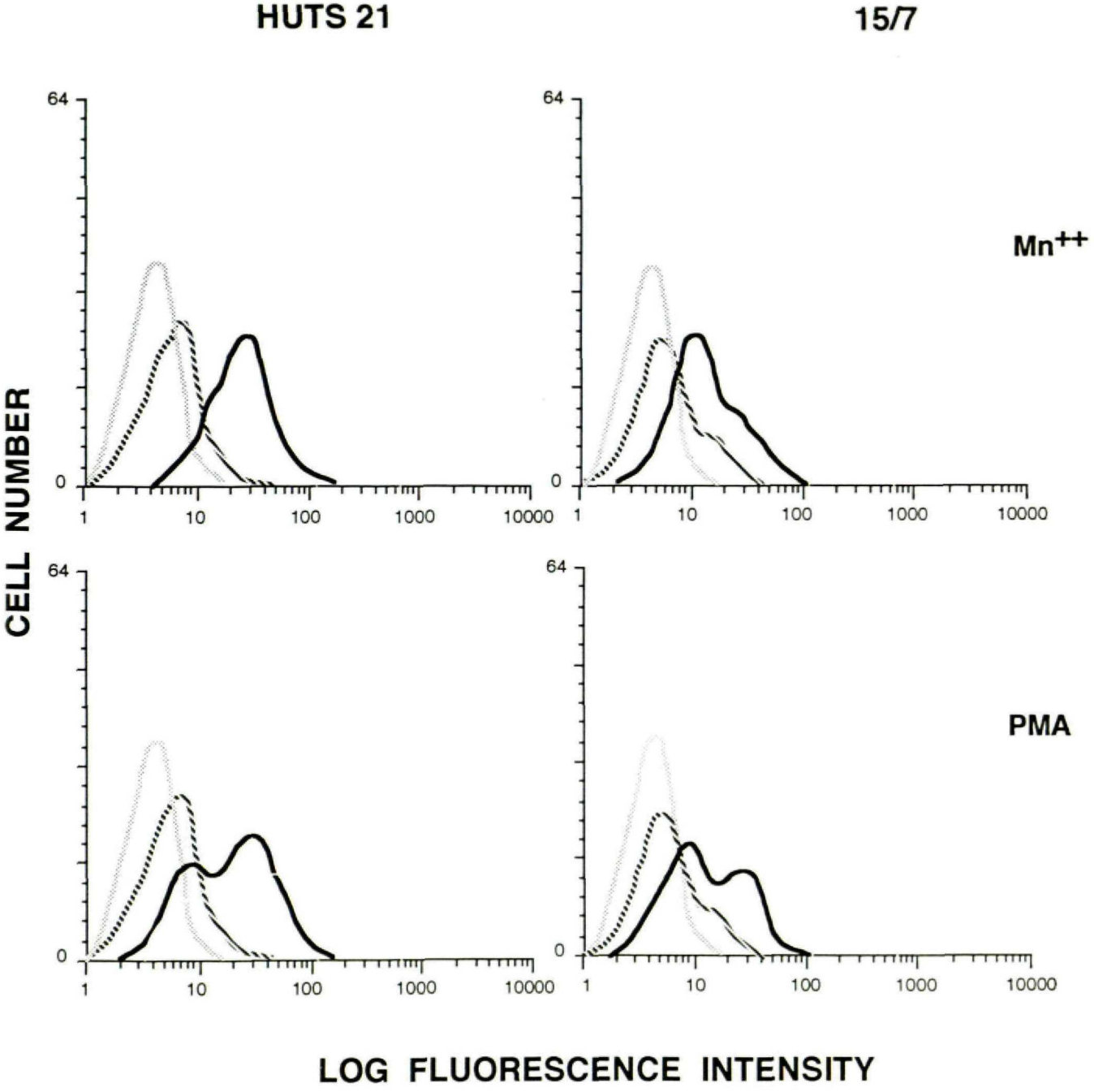

Figura 1. Expresión de epítopos conformacionales activados de integrinas β1 en linfocitos T de SP, tratados con PMA y Mn++. Las células T de SP estimuladas (línea continua) con PMA (20ng/ml) o Mn++ (500μM), y en condiciones basales (línea rayada), fueron teñidas con los AcMs 15/7 y HUTS 21 y analizadas por citometría de flujo. Los histogramas muestran el aumento de expresión de ambos epítopos tras la activación *in vitro* y la escasa expresión en células T en reposo, como demuestra la gran superposición con el control negativo X63 (línea punteada).

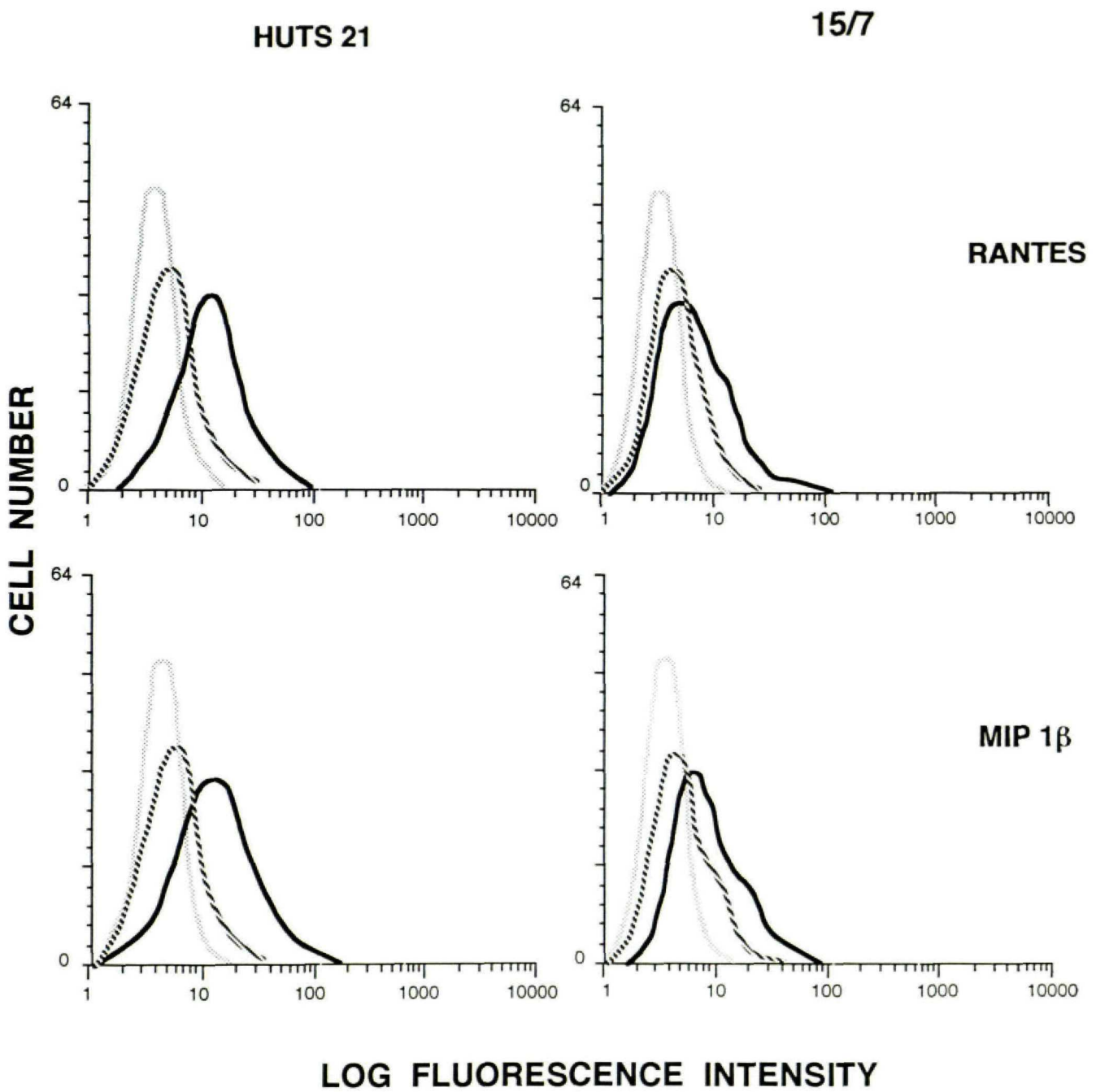

Figura 2. Regulación por quimioquinas de la expresión de conformaciones activas de la integrina β1 en linfocitos T de SP. Las células T estimuladas con las quimioquinas (línea continua) RANTES y MIP1β (0,1ng/ml) y en condiciones basales (línea rayada) fueron teñidas con los AcMs 15/7 y HUTS 21 y analizadas por citometría de flujo. Ambas quimioquinas indujeron una alta expresión de los dos epítopos conformacionales activados. La línea de puntos corresponde a la expresión del control negativo X63.

Quiero agradecer al Dr. A. Laffón y al Dr. A Postigo su permiso para incluir en esta tesis las publicaciones del Capítulo II y III de Resultados.

DISCUSION

La AR es una enfermedad inflamatoria crónica, caracterizada en su fase establecida por hipertrofia del revestimiento sinovial, infiltrados mononucleares de predominio linfocitario y neovascularización, que conjuntamente producen un crecimiento invasivo en el cartílago y hueso de la articulación afecta. Existe un consenso más o menos generalizado, de que el inicio de la enfermedad es un proceso inmunológico mediado por células T en respuesta a un antígeno específico, aunque desconocido. Sin embargo, es probable que el mantenimiento o perpetuación de la sinovitis crónica incluya un eje funcional célula T/macrófago/fibroblasto, en el que participen respuestas de activación celular T que pueden ser antigeno-indepedientes, junto a macrófagos y fibroblastos activados, responsables de la formación del *pannus* sinovial. Las evidencias acumuladas sobre la variación dinámica en el repertorio del RCT (Receptor de la Célula T), las teorías de los "superantígenos" y la posibilidad de antígenos perpetuadores en la propia articulación (colágeno tipo II, proteoglicanos del cartílago, *Heat-shock proteins*, etc), inapropiadamente reconocidos como heterólogos, podrían explicar el mantenimiento de la respuesta inmune celular (Panayi 1993; Lanchsbury y Pitzalis,1993; Firenstein 1992). Una vez desencadenada la respuesta inmune celular, es muy probable que se requiera el aporte constante de células inmunes desde el torrente circulatorio para mantener el proceso inflamatorio. Esto explicaría la exhaustiva vascularización de la MS hiperplásica y sugiere que la enfermedad podría ser alterada si se frenara el influjo de células inflamatorias. Existen dos hechos que podrían cooperar en la persistencia del infiltrado inflamatorio sinovial: 1) la existencia de vías de activación celular antígeno-independientes y 2) con independencia del antígeno desencadenante, el caracter crónico de la infiltración por células mononucleares puede venir determinado por factores que gobiernan el tráfico célular. La composición, distribución y volumen de este tráfico depende en gran medida de las interacciones adhesivas de las células mononucleares circulantes con a) las células endoteliales de las vénulas postcapilares y b) las células perivasculares y componentes de la MEC. Por lo tanto son las moleculas de adhesion expresadas en los diferentes tipos celulares las que van a determinar el reclutamiento no antigeno-específico de la mayoría de los linfocitos T. La expresión de receptores en los linfocitos T viene determinada por la activación y diferenciación celular, y la

expresión de sus ligandos en las CE y células tisulares (Ej. STF) se regula fundamentalmente por citoquinas. En la MS inflamada, estas citoquinas se liberan por las células perivasculares estimuladas inmunológicamente. Puesto que las interacciones entre las MA del linfocito T y sus ligandos celulares o de MEC son independientes de la especificidad inmunológica de la célula T, estas interacciones van a suponer un factor crítico en el desarrollo de la policlonalidad T dominante en los infiltrados inflamatorios sinoviales. Aunque las células T no hayan sido *especificamente* reclutadas, su papel patógenico sigue siendo relevante por la posibilidad de interacción con otras células y con la MEC que pueden conllevar señales activadoras y efectoras.

Dentro de las MA, los receptores de la familia VLA o integrinas β1 representan la clase predominante de receptores de MEC en linfocitos, y algunos de ellos como VLA-4, son capaces de soportar interacciones célula-célula. En el presente estudio se demuestra que varios heterodímeros VLA (α1-5, β1) están ampliamente distribuidos en la MS de pacientes con AR. La tinción inmunohistoquímica tisular señala la presencia de las subunidades α1, 2, 3 y 5 en las células del revestimiento sinovial y en CE, mientras que la mayoría de las células presentes en el infiltrado mononuclear son α4+ y un porcentage menor expresan α1 y α5. El AcM anti-β1 tiñe intensamente los sinoviocitos y vasos sanguíneos, con una reacción menos intensa pero difusa en los linfocitos. Las células T del LS muestran un patrón de expresión muy parecido, con aumentos de expresión significativos de α1, α4 y β1 respecto a sus correspondientes células T de SP. Sin embargo, es importante reseñar que la expresión de VLA-α5 es muy similar en los 2 compartimentos. Estos resultados son consistentes con los encontrados en pacientes con otras artropatías inflamatorias crónicas (García de Vicuña, datos no mostrados).

Es bien conocida la presencia de marcadores de activación en gran cantidad de células T de los compartimentos sinoviales : el antígeno HLA DR está presente en la mayoría de ellas (Burmester et al, 1981), y el antígeno de activación tardía VLA-1 se ha descrito en un subgrupo de células T del LS (Laffón et al, 1989) y MS (Cush y Lipsky, 1988), en concordancia con los datos de nuestro trabajo. Sin embargo, ninguno de estos dos antígenos son los más apropiados como marcadores de activación de células T, ya que se expresan en una gran cantidad de tipos celulares

tanto en reposo como activados (Ishikawa y Ziff, 1976; Hemler 1990). En este sentido, el antigeno de activación de células humanas CD69 ó AIM (activation inducer molecule) ha sido bien caracterizado estructural y funcionalmente, y se expresa tempranamente en células T y B bajo diferentes estímulos (Sánchez-Mateos et al, 1989). Además, disponíamos de un AcM específico para el heterodímero gp95/85, similar bioquímica y funcionalmente al antígeno de activación de células linfoides Ea2, previamente descrito (Newman et al, 1986). Las células T, tanto de LS como de MS, de todos nuestros pacientes con AR mostraron una alta expresión de los dos marcadores de activación AIM y gp85/95, comparado con la ausencia o baja expresión en la superficie de las células T de SP correspondientes o de controles sanos.

Por lo tanto, los linfocitos T que infiltran la MS y pasan a formar parte del LS contituyen una población de células activadas que además muestran una expresión incrementada de receptores VLA-1 y fundamentalmente VLA-4.

REGULACION DE LAS INTERACCIONES DE LINFOCITOS T DE AR CON FIBRONECTINA

El primer nivel de regulación de la adhesión de las células a componentes de MEC viene determinado por la expresión en superficie de los receptores apropiados y por la densidad molecular de estos receptores. Sin embargo, la presencia del receptor no presupone actividad funcional y concretamente el estudio de los linfocitos en reposo revela una adhesión mínima a proteínas de MEC aunque la expresión del receptor o receptores sea relevante. Los linfocitos T utilizan el receptor VLA-5 ($\alpha 5\beta 1$) para unirse a la secuencia RGD en el dominio central de unión a células de la FN humana y que está contenido en un fragmento proteolítico de 80kD. Además pueden unirse a FN a través del receptor VLA-4 ($\alpha 4\beta 1$), en una región distinta no dependiente de RGD, y presente en un fragmento de 38 kD. Nosotros hemos encontrado una escasa población de células T en SP capaces de unirse a los fragmentos proteolíticos de la FN humana tanto de 80 kD como de 38 kD, a pesar de la presencia de los receptores VLA-4 y VLA-5 en estas células. Por el

contrario, una proporción significativamente mayor de células en el LS fue capaz de unirse a cada fragmento correspondiente de FN.

Existen dos posibles explicaciones para las diferencias de adhesión a los dos fragmentos de FN entre células T de SP y LS. Por un lado, la expresión de VLA-4 está aumentada en las células del LS y se expresa en una población mayor que en SP, lo que podría explicar la mayor adhesión al fragmento de 38 KD. La proporción de células T de SP que se adhieren a FN a través de VLA-4 está dentro del rango descrito para células T de SP de controles sanos y se correlaciona con el porcentaje de células T de memoria (Shimizu et al, 1990). Nosotros hemos encontrado también una correlación entre el porcentaje de adhesión a FN y la expresión del antígeno CD45RO de células T de memoria, en los dos compartimentos. El análisis fenotípico de las células de memoria circulantes demuestra que este subgrupo expresa niveles mayores de VLA-4, VLA-5 y VLA-6, junto con otras moléculas de adhesión, y esto se correlaciona con una capacidad aumentada de unión a sus ligandos FN y LM (Shimizu et al, 1990). Nuestros resultados corroboran hallazgos previos de otros autores (Pitzalis , 1987; Hanly, 1990) de la presencia del marcador CD45RO en la mayoría de las células T activadas del LS y MS (Capítulos II y III de resultados), y están en consonancia con estudios *in vitro* que demuestran la adquisición del antígeno CD45RO tras la activación de la célula T (Akbar et al, 1988). Todos estos datos explicarían el aumento de receptores VLA-4 en el compartimento sinovial, que deben ser receptores funcionalmente activos, puesto que se correlacionan con adhesión incrementada. Sin embargo, el aumento de adhesión de las células del LS a través de VLA-5 (45% de media) frente a las de SP (19% de media) no puede explicarse completamente por incrementos en la población $\alpha 5+$, ni por aumentos, insuficientes, en su expresión. Estos hallazgos sugieren un segundo mecanismo que conlleva un aumento de la función de la integrina VLA-5 mediada por la activación *in vivo* de las células T, y que presumiblemente también actúa en los receptores VLA-4. En este sentido, las células T en reposo que apenas se unen a FN y LM a pesar de niveles detectables de sus receptores, incrementan dramáticamente su adhesión a los pocos minutos del tratamiento con PMA, AcMs anti-CD3/RCT o AcMs activadores anti-CD2 (Shimizu et al., 1990). Este cambio rápido en la adhesión a FN o LM tras la activación de la célula T, ocurre

sin cambios detectables en la expresión de VLA-4, VLA-5 y VLA-6, lo que sugiere un cambio cualitativo probablemente mediado por un cambio conformacional en la integrina preexistente. En este contexto, se han descrito cambios conformacionales de otras integrinas β2 y β3 durante la activación *in vitro* que favorecen las interacciones con sus respectivos ligandos (Hynes, 1992). Existen otras evidencias de la modulación de la integrina VLA-5 en queratinocitos epidérmicos, donde la pérdida de la capacidad de adhesión a FN a través de VLA-5 precede en varias horas a la pérdida de la integrina de la superficie celular (Adams y Watt, 1990).

Existen otros mecanismos potenciales de regulación de las interacciones linfocitarias con FN que vienen determinados por la heterogeneicidad en la estructura de la proteína, y que pueden ser especialmente relevantes *in vivo* . Por ejemplo, la secuencia de reconocimiento de VLA-4 en la FN se encuentra en una región generada por procesamiento alternativo de la molécula (Yamada 1991a). Nos podemos encontrar con MECs que difieran en la densidad de fragmentos CS-1. La distribución anatómicamente restringida de estas formas de FN *in vivo* podría tener efectos significativos en la capacidad de los linfocitos para unirse a FN via VLA-4. En este contexto, estudios inmunohistoquímicos indican que el fragmento CS-1 se expresa selectivamente en el tejido sinovial de AR pero no en la MS normal (Elices et al., 1994). En contraste con otras formas de FN de la MEC tisular, la expresión de CS-1 se limita a la vasculatura y a los sinoviocitos del *lining*. El fragmento CS-1 se localiza en la superficie luminal de las CE y es capaz de mediar la adhesión de células linfoides intravasculares que expresan formas funcionalmente activas de VLA-4, pero no de células con receptores inactivos (Elices et al., 1994). Estos hallazgos nos permiten especular sobre la importancia de las interacciones VLA-4/CS-1 en el reclutamiento de linfocitos desde el torrente vascular a la MS inflamada, ya que las CE son capaces de sintetizar FN bajo estímulos proinflamatorios. Las interacciones de células T, a través de VLA-5, con otras formas de FN que contienen el fragmento RGD, presentes en la MEC tisular, podrían suponer el anclaje para las células que ya han conseguido llegar a la articulación. Recientemente, se ha demostrado la capacidad de péptidos sintéticos de FN que contienen las secuencias RGD o CS-1 para bloquear el

reclutamiento de células mononucleares a la articulación en un modelo experimental de artritis en ratas, constatando la supresión de la fase crónica de sinovitis (Wahl et al., 1994).

Se han encontrado niveles aumentados de FN en el LS de pacientes con AR, lo que parece traducir un aumento de la producción local en la articulación. Es más, la producción de FN y otras proteínas de MEC (COL, LM, entactina, etc) se ha demostrado a nivel ultraestructural en STF de la capa bordeante (Revel et al., 1995). La distribución pericelular de estas proteínas en torno a los STF, desde la capa más profunda de la íntima sinovial, tiene problamente una función de anclaje para los sinoviocitos y para otras células con receptores adecuados para MEC. Es muy probable que la presencia de CS-1 en los sinoviocitos del revestimiento sinovial juegue un papel importante en la interacción con otras células del tejido portadoras de VLA-4 como monocitos y linfocitos, capaces además de interaccionar con VCAM-1. La preponderancia de uno u otro ligando de la integrina VLA-4, dependiendo de cambios en el microambiente sinovial, determinará el tipo de interacción.

Por último, una cuestión crítica en la adhesión de células T a FN es la capacidad para transmitir señales que resulta de la interaccion ligando-receptor. Es bien conocido el efecto comitogénico de la FN a través de VLA-4 y VLA-5 con la señalización vía CD3 en linfocitos T (Davis y Lipsky, 1993). La interacción de células CD4+ con FN es capaz de inducir la expresión del factor de transcripción AP-1 implicado en la regulación de la expresión del gen de IL-2 (Yamada et al., 1991b). En otros tipos celulares, la unión a proteínas de MEC a través de receptores β1 puede inducir la expresión de genes de metaloproteasas (fibroblastos, condrocitos) o cambiar los patrones de expresión génica para enzimas, citoquinas o quimioquinas (monocitos) (Juliano y Haskill, 1993; Arner y Tortorella, 1995)

La presencia de varios receptores de la familia VLA en múltiples tipos celulares de la MS y la localización tisular crítica de moléculas de MEC en la sinovial y el cartílago de la AR, pueden conducir a un entendimiento mejor de los mecanismos de destrucción tisular.

REGULACION DE LA ADHESION DE LINFOCITOS T DE AR A LAS MOLECULAS ENDOTELIALES VCAM-1 Y ELAM-1.

La migración de linfocitos desde la SP a la MS requiere su unión previa al endotelio vascular. En situaciones de migración celular a tejidos inflamados los diferentes patrones de citoquinas o mediadores inflamatorios conducen a la inducción diferencial de unos ligandos endoteliales u otros. En los vasos de la MS reumatoide, donde citoquinas como TNFα e IL-1 son abundantes, se ha encontrado una expresión incrementada de los dos ligandos endoteliales VCAM-1 y ELAM-1 (Koch et al., 1991). Su expresión es más frecuente que la encontrada en artrosis y en el caso de ELAM-1, su expresión se correlaciona con la actividad inflamatoria de la AR (Kriegsmann et al., 1995). Ambas moléculas pueden participar en el primer paso de la adhesión al endotelio independiente de la activación linfocitaria, y VCAM-1 media además la adhesión firme de los linfocitos tras su activación (Ver párrafo 4. de la Introduccion).

Nuestros resultados demuestran que las células T de LS y MS tienen mayor capacidad para adherirse a ELAM-1 que sus correspondientes de SP de los pacientes o de controles sanos. La mayoría de las células T sinoviales expresan el marcador de células T de memoria CD45RO, y en este sentido, se ha descrito que ELAM-1 media exclusivamente la adhesión de células T de memoria y no depende de la activación celular (Shimizu et al., 1991a; 1991b). Asimismo, un subgrupo de células T circulantes de memoria en pacientes con enfermedades inflamatorias crónicas de la piel son la población mayoritaria que interacciona con ELAM-1, y se distinguen por la presencia del Antígeno Cutáneo Asociado (Picker et al., 1991). Estas células contituyen el 80-90% de las células T infiltrantes en las dermatitis crónicas, pero menos del 5% en los tejidos extracutáneos de inflamación crónica. Queda por determinar si la población de células T capaces de interaccionar con ELAM-1 en AR presenta una restricción específica similar. Aunque desconozcamos el ligando linfocitario, la interacción de las células T tanto de SP como de compartimentos sinoviales es específica, ya que se inhibe únicamente con AcM anti-ELAM-1.

La identificación de VLA-4 como ligando de VCAM-1 (Elices et al., 1990) sugirió que esta vía de adhesión podría tener un papel crucial en la migración de las células que lo expresan desde la sangre a los tejidos inflamados, y así lo han confirmado múltiples estudios posteriores (Postigo et al., 1993; Lobb y Hemler, 1994). AcM específicos frente a la subunidad $\alpha 4$ y frente a VCAM-1 se han utilizado satisfactoriamente en el tratamiento de diferentes enfermedades inflamatorias experimentales. Un buen ejemplo es la encefalomielitis autoinmune experimental, en la que, estos anticuerpos, inhiben el progreso de la enfermedad al interferir la migración de leucocitos al parénquima cerebral (Yednock et al., 1992). En el presente estudio demostramos que la mayoría de las células T del LS y MS son capaces interaccionar con VCAM-1 a través de la integrina VLA-4, frente a una población minoritaria en SP. Este aumento de adhesión podría estar en relación con el fenotipo activado y de memoria predominante en los compartimentos sinoviales, ya que la interacción VLA-4/VCAM-1 es poco relevante en las células T vírgenes y aumenta *in vitro* tras la activación de la célula T (Shimizu et al., 1991b).

Nuestros datos sugieren la existencia de varios niveles de afinidad de la integrina VLA-4 por sus ligandos. La capacidad de adhesión aumentada de las células T sinoviales a VCAM-1 y FN 38KD refleja un aumento de afinidad inducido *in vivo*. Sin embargo, podemos aumentar la adhesión de las células del LS a sus dos ligandos mediante el tratamiento *in vitro* con ésteres de fórbol o a través de señales transmitidas por el complejo CD3/RCT. Estos resultados sugieren cambios cualitativos en las integrinas VLA-4 con diferente capacidad para unirse a sus ligandos.

Tanto ELAM-1 como VCAM-1 podrían participar en diferentes pasos de adhesión a endotelio de las células T de SP para alcanzar la MS. Podemos establecer un modelo hipotético que resuma la contribución de ambas moléculas. Un subgrupo de células T de memoria circulantes podría interaccionar con ELAM-1 inducido tempranamente en el endotelio sinovial por el estímulo inflamatorio. La inducción de VCAM-1 podría además facilitar la *captura* y el rodamiento de linfocitos circulantes aunque no presentasen un supuesto ligando específico para ELAM-1, aumentando así la eficacia del endotelio para reclutar células al tejido. Los linfocitos ya detenidos sobre la pared del vaso podrían ser activados por citoquinas y quimioquinas locales conduciendo a

la activación de las integrinas β1 (VLA-4) y β2 (LFA-1). En este sentido, se ha descrito que la quimioquina MIP 1β inmovilizada sobre proteoglicanos, es capaz de aumentar la interacción de VLA-4 con su ligando VCAM-1 (Tanaka et al., 1993). Es también posible que la interacción inicial mediada por ELAM-1 potencie la adhesión posterior mediada por integrinas. En relación con este punto, la unión de neutrófilos a ELAM-1 desencadena la activación de Mac-1 y aumenta la adhesión al endotelio (Lo et al, 1991) Las integrinas activadas establecerían la adhesión firme y subsiguiente diapedesis mediante las interacciones VLA-4/VCAM-1 y LFA-1/ICAM-1. Apoyando nuestros hallazgos, otros autores han descrito la relevancia de la interacción VLA-4/VCAM-1 en la adhesión de linfocitos T de SP a vasos de MS de AR (van Dinther-Janssen et al., 1991). Aunque la técnica de adhesión *in vitro* a cortes de tejido utilizada por estos autores no está exenta de críticas, sus resultados muestran una adhesión más prominente a través de VCAM-1 que de ICAM-1. Sin embargo se han comunicado resultados discordantes respecto al papel funcional de VCAM-1 en el endotelio de AR. Otros autores sostienen que la adhesión de líneas linfoblastoides a los vasos de la sinovial de AR, también sobre cortes histológicos de MS, es inhibida fundamentalmente por péptidos CS-1 y escasamente por AcMs anti-VCAM-1 (Elices et al., 1994). Por lo tanto, defienden la preponderancia del fragmento CS-1, presente en la superficie luminal de las CE, en la adhesión de células linfoides al endotelio de AR, que requiere además interacción con receptores VLA-4 activados. Estos datos descartan la participación de este fragmento de FN en la interacción inicial de linfocitos con el endotelio, que sí se ha demostrado para VCAM-1 (Ver epígtrafes 3.2 y 4.1 de Introducción). Serían necesarios estudios para conocer si la presencia de fragmentos de FN en la superficie de las CE enmascara o dificulta de alguna forma la exposición de las moléculas de VCAM-1 presentes en el mismo endotelio. Por otro lado, dado que diferentes citoquinas y agentes proinflamatorios en la MS pueden regular tanto la aparición de moléculas endoteliales como el procesamiento alternativo de FN, las variaciones locales y temporales de estos factores en la MS de AR serían decisivos para la preponderancia de una u otra vía de adhesión.

Finalmente, la importancia de las interacciones VLA-4/VCAM-1 en linfocitos T de AR no se restringe al reclutamiento endotelial. La presencia de VCAM-1 en otros tipos celulares de la MS como macrófagos y sinoviocitos también es relevante. Previamente se ha descrito que las interacciones de VCAM-1 con VLA-4 son capaces de desencadenar respuestas proliferativas en linfocitos T de SP (Burkly et al., 1991). Nosotros hemos encontrado que esta interacción también desencadena respuestas proliferativas en células T del LS y esto podría representar un mecanismo importante para el mantenimiento de la respuesta inmune celular en la sinovitis crónica de la AR.

INTERACCION DE LINFOCITOS T DE ARTRITIS REUMATOIDE CON SINOVIOCITOS TIPO FIBROBLASTO Y SU REGULACION

Las interacciones de linfocitos T con células del revestimiento sinovial hiperplásico en la MS de AR son probablemente relevantes en los mecanismos destructivos del tejido invasivo sinovial. En el presente estudio hemos encontrado un considerable aumento de células T en LS y MS capaces de interaccionar con STF de AR, frente a una población muy escasa de células en SP. Las tres vias moleculares de adhesión estudiadas están involucradas en estas interacciones: CD2/LFA-3, LFA-1/ICAM-1 y VLA-4/VCAM-1, aunque esta última es particularmente prominente en las células T de compartimentos sinoviales. El tratamiento tanto de las células T de SP como de los STF con las citoquinas IL-4, TNFα, IFNγ y en menor medida IL-1β, es capaz de aumentar la adhesión de las células T de SP a STF. Por lo tanto, la adhesión aumentada de las células T de compartimentos sinoviales podría estar regulada *in vivo*, al menos en parte, por el efecto de citoquinas en las moleculas involucradas.

Sabemos que la expresión de las moléculas ICAM-1 y VCAM-1 está aumentada en los sinoviocitos del revestimiento sinovial de AR, respecto a MS normales (Koch et al., 1991). Esta expresión incrementada se debe probablemente a la gran concentración de citoquinas proinflamatorias presentes en la sinovial. La expresión constitutiva de ICAM-1 y VCAM-1 en STF de AR en cultivo puede incrementarse *in vitro* por IL-1β, TNFα e IFNγ y la citoquina IL-4 aumenta además la expresión de VCAM-1 (Krzesicki et al., 1991; Morales Ducret et al., 1992). La

expresión de LFA-3 no se afecta por citoquinas pero su expresión basal en STF es más alta (Chin et al., 1990). El análisis cuantitativo de citoquinas, tanto a nivel de proteína como de expresión génica, con las técnicas utilizadas hasta el momento, conducía a un consenso generalizado de que las citoquinas de origen macrofágico como IL-1 y TNFα se producían en grandes cantidades en la MS de AR, mientras que las de origen linfocitario como IL-2, IL-4 ó IFNγ eran virtualmente indetectables. Estudios recientes con una nueva técnica de tinción inmunohistoquímica intracelular han detectado la presencia de múltiples citoquinas derivadas de células T en la MS de pacientes con AR, y demuestran el origen de estas citoquinas en los linfocitos T (Ulfgren et al., 1995). Por otro lado, la presencia de citoquinas en tejidos inflamados no se produce aisladamente y es razonable pensar que su efectos simultáneos *in vivo* tengan importantes funciones moduladoras. Por lo tanto, aún admitiendo que IL-4 e IFNγ tuvieran una escasa representación, sus efectos simultáneos con otras citoquinas sobre STF podrían ser relevantes. En este sentido, se ha descrito que la combinación de IFNγ+TNFα y de IL-4+TNFα sobre CE en cultivo es más eficaz que TNFα aislado para estimular la adhesión de células T (Haskard y Thornhill, 1993). El efecto aditivo de IL-4 y TNFα en la adhesión linfocitaria aumentada está mediado fundamentalmente por el aumento sinérgico de VCAM-1 inducido en el endotelio por las citoquinas.

En nuestro sistema experimental, IL-1β tuvo efectos variables e indujo incrementos de adhesión menores de los esperados en relación a la inducción de moléculas que produce en los STF (Morales Ducret et al., 1992). Discrepancias entre la expresión y la función de moléculas inducidas por IL-1β, como ICAM-1, ya se habían descrito en fibroblastos dérmicos (Piela y Korn, 1990; Piela et al, 1991). Estos datos sugieren la existencia de mecanismos adicionales de regulación y la posibilidad de cambios cualitativos inducidos por la citoquina en la molécula ICAM-1.

El papel regulador de las citoquinas en la expresión de MA por el endotelio y otras células no linfoides está bien establecido, pero poco se conoce de su influencia en ligandos de células T. Nuestros datos demuestran que las células T de SP tratadas con IL-4, TNFα e IFNγ, tienen mayor capacidad de adhesión a STF que las no estimuladas. Sin embargo los escasos cambios en el nivel de expresión de las moléculas implicadas no se correlacionaron con el nivel de

adhesión inducido por las mismas citoquinas. Estos resultados podrían explicarse por la existencia de cambios cualitativos, además de los posibles cuantitativos, producidos por las citoquinas en las MA linfocitarias. Hemos comprobado este efecto de las citoquinas TNFα e IFNγ en la inducción de cambios conformacionales activados de la integrina β1, que se correlaciona con una capacidad aumentada de las células T estimuladas para unirse a FN. Aunque estos hallazgos serán discutidos más adelante, es interesante recordar que estos epítopos conformacionales activados prácticamente ausentes en células T de SP, se han podido detectar en los linfocitos del LS y la MS de AR. Por lo tanto, es razonable pensar que, al menos las interacciones dependientes de VLA-4, puedan ser reguladas *in vivo* por citoquinas.

Existen otras posibles explicaciones para el diferente comportamiento funcional entre los linfocitos de SP y compartimentos sinoviales, y que ya hemos aducido previamente para las interacciones con MEC y con ligandos endoteliales. Prácticamente el 100% de las células de SP y del compartimento sinovial expresan las integrinas LFA-1 y VLA-4 con sus 2 cadenas α y β, y la molécula CD2. Sin embargo, las células T del compartimento sinovial presentan mayor densidad de estas moléculas sobre su superficie, como corresponde al fenotipo predominante de células T de memoria. Otros estudios exclusivamente sobre células T de SP, han descrito que la población T que se une a STF estimulados con IL-1β e IFNγ es minoritaria en la expresión del marcador de células vírgenes CD45RA, y presenta una mayor expresión de LFA-1, CD29 (β1) y CD2 (Matsuoka et al., 1991). Por lo tanto, este fenotipo parece corresponder al subgrupo de células T de memoria de SP, aunque los autores no hayan determinado el marcador CD45RO.

Sin embargo, los aumentos de expresión de las moléculas linfocitarias, detectados por citometría de flujo en las células del compartimento sinovial, no fueron suficientes en la mayoría de los casos para explicar la adhesión tres o cuatro veces superior respecto a células de SP. Los tres receptores linfocitarios involucrados en la adhesión a STF, CD2, LFA-1 y VLA-4 sufren cambios cualitativos hacia conformaciones funcionalmente activas tras la activación de la célula T (Shimizu et al ., 1990; Makgoba et al., 1992). Puesto que las células T de LS y MS están activadas, la

activación de los receptores podría explicar el aumento de adhesión que no es capaz de sustentarse únicamente en el aumento del número de receptores.

Finalmente, la importacia de las vías moleculares de adhesión demostradas en las interacciones de linfocitos T de AR con STF se refuerza por su capacidad para la transmisión de señales. La molécula LFA-3 puede funcionar como señal coestimuladora para CD2 y promover la estimulación fisiológica de la célula T en ausencia de antígeno (Dustin et al., 1989a; Bierer et al 1989). Las moléculas LFA-1 y VLA-4, así como sus ligandos ICAM-1 Y VCAM-1, pueden transmitir señales sinérgicas con CD3/RCT para inducir la proliferación de la células T (Davis y Lipski 1993; Burkly et al., 1991). LFA-3 en monocitos y células epiteliales tímicas parece estar involucrado en la producción y liberación de IL-1 (Haynes et al., 1989). Por lo tanto, la adhesión de la célula T a células accesorias a través de CD2/LFA-3 podría resultar en un aumento de la producción de citoquinas por estas células.

Las interacciones celulares mediadas por estas tres vías de adhesión podrían tener un papel relevante *in vivo* en las funciones efectoras de linfocitos T y sinoviocitos. Apoyándonos en los datos obtenidos *in vitro,* es razonable pensar que las citoquinas proinflamatorias, tan abundantes en el tejido sinovial de la AR, pueden estar regulando estas interacciones en cada tipo celular por diversos mecanismos.

PRESENCIA DE CONFORMACIONES ACTIVAS DE LAS INTEGRINAS β1 EN AR Y SU REGULACION POR CITOQUINAS Y QUIMIOQUINAS

Hasta ahora hemos visto que la capacidad de las células T obtenidas del LS y de los infiltrados inflamatorios de la MS de AR, para adherirse a proteínas de MEC o ligandos celulares a través de integrinas VLA, está aumentada respecto a las células de SP. Estos datos suponen la existencia de una regulación de los receptores VLA/β1 que tiene lugar *in vivo* y que parece íntimamente ligado al estado de activación de las células T. La activación de las integrinas β1 puede inducirse *in vitro,* desde el exterior celular, con AcM activadores como TS2/16 o cationes

divalentes como Mn^{++}, pero también por señales intracelulares generadas por la activación celular con ésteres de forbol o a través de moléculas coestimuladoras como CD2 y CD3 (Ver epígrafes 3.3.3 y 3.3.4. de la Introducción). Por lo tanto, la activación de las células T *in vivo*, por mecanismos tanto antígeno-dependientes como antígeno-independientes, puede conducir a la activación de las integrinas con el consiguiente cambio de afinidad por sus ligandos. De hecho, se ha descrito la expresión coordinada de epítopos de activación de integrinas β1 y β2 durante la activación de las células T en órganos linfoides secundarios (Picker et al, 1993). Sin embargo, la expresión de conformaciones activas de la integrina β1 en condiciones de respuesta inmune alterada no había sido investigada hasta el momento. El presente estudio demuestra la presencia de estas conformaciones activas en el infiltrado inflamatorio sinovial de la AR, así como en otras enfermedades inflamatorias crónicas como la tiroiditis autoinmune de Hashimoto o el síndrome de Sjögren (datos en glándulas salivares no mostrados).

El epítopo activado 15/7, ausente o con mínima expresión en las células T de SP tanto de pacientes como de controles sanos, está claramente aumentado en las células T del LS de los pacientes con AR y otras artropatías inflamatorias crónicas. Un hallazgo interesante es la capacidad de las citoquinas TNFα e IFNγ de inducir la expresión de este epítopo en las células T de SP, ya que se trata de reguladores fisiológicos que están presentes en el microambiente de la sinovial inflamada. Estos datos apoyan además nuestros resultados del aumento de adhesión a STF inducido por citoquinas en los linfocitos T de SP.

Los datos acumulados durante los últimos años, han revelado el papel de las citoquinas como excelentes candidatos para *estimular fisiológicamente* la adhesión (Zimmerman et al., 1992; Tanaka et al., 1993; Springer 1994). Son liberadas en lugares de inflamación, actúan a distancia y son capaces de inducir infiltrados inflamatorios *in vivo*. Aunque algunas citoquinas promueven la adhesión incrementando el nivel de expresión de superficie de receptores de adhesión o sus ligandos, otras, por diferente mecanismo, incrementan la afinidad del receptor rápidamente. Aunque estas citoquinas pro-adhesivas no están restringidas a una sóla familia, la familia de las quimioquinas reviste especial importancia (ver Introducción, epígrafe 4.2). Por ejemplo, MIP-1β

puede promover la adhesion de ciertos grupos de linfocitos a VCAM-1 y a FN a través de la activación de integrinas β1 (Tanaka et al., 1993a), e induce quimiotaxis de células T activadas CD4+ (Schall et al., 1993). RANTES induce la migración de células T de memoria activadas y no activadas (Schall et al., 1990). La presencia de RANTES, a nivel de proteína, y de su receptor han sido detectados en la sinovial de AR (Snowden et al., 1994), aunque el receptor detectado es común para otras quimioquinas como MIP-1α y β (Whyte y Binns, 1994). Por lo tanto, nuestros resultados de la presencia de epítopos conformacionales activados de β1 en la MS, y la inducción *in vitro* de estos epítopos en linfocitos por RANTES y MIP-1β, apoyan la hipótesis de que estas quimioquinas intervengan en fenómenos adhesivos de linfocitos a endotelio y probablemente a MEC en la AR.

La presencia de integrinas β1 activadas, tanto en AR como en otras enfermedades inflamatorias crónicas, parece tener además consecuencias funcionales en las células linfoides que lo expresan. La localización selectiva del epítopo 15/7 en los contactos de adhesión célula-sustrato, y el efecto amplificador del AcM 15/7 en la adhesión a FN a través de VLA-5, demuestran la importancia de este epítopo en la adhesión celular. La inducción de adhesión y de cambios morfológicos como el *spreading* celular en un sustrato de FN, sugieren que este epítopo puede estar implicado en la trasmisión de señales intracelulares que afecten al citoesqueleto celular.

En resumen, nuestros resultados describen por primera vez la presencia de conformaciones activas de integrinas β1 en tejidos crónicamente inflamados, y su aparición en linfocitos T puede ser regulada por mediadores fisiológicos como citoquinas. La modulación de la adhesión y morfología celular a través de estos epítopos, bajo la influencia de quimioquinas, puede estar contribuyendo decisivamente a la formación del foco inflamatorio. La pregunta obligada es si la expresión preferencial de estos epítopos en tejidos de inflamación crónica, puede convertirlos en la diana adecuada para la intervención terapéutica.

CONCLUSIONES

1) Las integrinas β1, VLA1-5, se expresan ampliamente en sinoviocitos, vasos y células mononucleares de la MS reumatoide. Los linfocitos del infiltrado, al igual que las células T del LS, expresan VLA-1, VLA-5 y, fundamentalmente VLA-4.

2) Los linfocitos T de compartimentos sinoviales, que portan los marcadores de activación CD69 y gp95/85, exhiben una capacidad de adhesión aumentada a dos lugares de unión de la FN (RGD y CS-1), respecto a sus correspondientes de SP. Este aumento de adhesión puede estar mediado por un aumento de receptores VLA-4 y por la activación de ambos receptores VLA-4 y VLA-5 en las células T sinoviales.

3) Los linfocitos T de compartimentos sinoviales, mayoritariamente con fenotipo activado y de memoria, muestran una adhesión aumentada a los ligandos endoteliales ELAM-1 y VCAM-1 respecto a células T de SP. La interacción de VCAM-1 con VLA-4 es capaz, además, de transmitir señales coestimuladoras a linfocitos T de SP y LS.

4) La adhesión de linfocitos T de LS y MS a STF, está aumentada respecto a la población de SP, e involucra, al menos, tres vías moleculares de adhesión: LFA-1/ICAM-1, VLA-4/VCAM-1 y CD2/LFA-3. Además del aumento de receptores en STF, las citoquinas regulan estas interacciones *in vitro* mediante la inducción de cambios cualitativos en los ligandos de linfocitos T de SP.

5) La presencia *in vivo* de conformaciones activas de integrinas β1 en la MS de AR y tejidos de enfermedades inflamatorias crónicas, se demuestra formalmente mediante AcM específicos que reconocen epítopos dependientes de activación de la integrina β1. La expresión de estos epítopos, prácticamente ausentes en células T de SP, puede ser inducida o aumentada *in vitro* por factores activadores, citoquinas y quimioquinas. Además, estos epítopos son capaces de inducir cambios morfológicos en la célula, así como un aumento de adhesión.

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